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INFLUENCE OF GESTATIONAL AGE AND HORMONES ON EXPERIMENTAL FOETAL BRADYCARDIA

BY J. D. MARTIN* AND I. MAUREEN YOUNG

From the Department of Obstetrics and Gynaecology and The Sherrington School of Physiology, St Thomas's Hospital Medical School, London, S.E. 1

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A single intravenous injection of adrenaline or noradrenaline into the maternal circulation causes a transient bradycardia of the exposed foetus in the near-term rabbit and guinea-pig (Dornhorst & Young, 1952); this was shown to be due to asphyxia consequent upon the reduction in maternal placental blood flow caused by constriction of the uterine arteries, and not due to any accompanying activity of the uterine muscle. The purpose of the present investigation was to study the influence of gestational age on this asphyxial bradycardia, in the guinea-pig foetus *in utero*. It was found that vasoconstrictor drugs in the maternal circulation caused slowing of the foetal heart at the end of gestation only; the experimental evidence suggests that this is not due to a greater sensitivity of the foetal heart to asphyxia, but to a hormonal sensitization of the maternal placental vessels to vasoconstrictor substances. The possible clinical significance of the findings is discussed.

METHODS

The observations were made on 75 guinea-pigs at gestational ages ranging from the fortieth day of pregnancy to term (67 days). The approximate age of the foetuses was determined before the experiment by palpation and afterwards the crown–rump lengths were measured; foetal ages will be described in these terms, and when reference is made to an approximate age this has been estimated from the values for crown–rump length given by Draper (1920) and Bell (1941).

Light anesthesia was induced and maintained with pentobarbitone sodium (Nembutal, Abbott Laboratories) 20–30 mg/kg, given into the brachial vein under local procaine injection. The maternal arterial pressure was recorded from a saline-filled polythene catheter (No. 3) in the carotid artery. The maternal abdominal wall was opened by a mid-line incision, under saline at 38° C, and the exposed uterus supported on a gauze sling. Intra-amniotic pressure was measured from a saline-filled polythene catheter (No. 2) inserted through the intact uterine wall and membranes. Both pressures were measured with New Electronic Products inductance type manometers and the recordings, together with the foetal e.c.g., were made with the N.E.P. recorder through pencil-type mirror galvanometers with an ultra-violet light source and print-out recording paper.

* Present address: Obstetric Unit, University College Hospital Medical School, London, W.C. 1.

The foetal heart rate was counted over periods of 7·5 or 15 sec, from e.c.g. records obtained with the uterine wall intact. Two No. 1 serum needles were inserted, 0·5 cm. apart, well into the foetal thorax, where the heart could be palpated; in order to get a good impulse free from interference, it was essential to earth all the apparatus used.

Vasoconstrictor and other drugs were injected into the maternal jugular vein, cannulated with No. 1 polythene tubing. Each was diluted with NaCl solution 0·9 % (w/v) and given in volumes of 0·25–1·0 ml., followed by 0·5 ml. saline. The vasoconstrictor drugs studied were adrenaline tartrate (British Drug Houses), noradrenaline (Levophed, Bayer Products), serotonin (Roche), vasopressin and ergometrine (Burroughs Wellcome). Oxytocin (Syntocinon; Sandoz, and Pitocin; Parke, Davis) was used to stimulate the uterine muscle and dibenzyline (Smith, Kline & French) was used for its anti-adrenaline properties.

The sensitivity of the foetal heart to hypoxia was studied by administering gas mixtures containing low oxygen tensions to the mother; mixtures of O_2 10 % in nitrogen and O_2 6 % in nitrogen were used, delivered from Douglas bags, at the rate of 1–2 l./min to a half rubber balloon which fitted closely over the maternal snout.

The influence of the ovarian hormones on the sensitivity of the uterine maternal placental vessels to vasoconstrictor drugs was investigated after the subcutaneous injection, 24 hr previously, of either 10 mg progesterone (Proluton; British Schering) or 1 mg oestradiol monobenzoate (Oestroform; British Drug Houses).

RESULTS

The extent and form of the electrical variations recorded from the foetal heart were variable and had no particular significance, since the electrodes were placed so as to obtain the best record with no regard to the orientation of the heart. The QRS complex showed a deflexion of 0·5–1·0 mV in good records; the T wave was frequently inverted during asphyxia. Figure 1 shows that the records obtained were uninfluenced by the maternal heart, the foetal heart slowing only during the administration of the vasoconstrictor drug to the mother. Bauer (1938) also used the e.c.g. to measure the foetal heart rate in the rabbit; the method has the advantage that the foetus may be studied *in utero*.

The resting foetal heart rates were variable, ranging from 160 to 320/min, the faster heart rates usually being observed in the more mature foetuses (Fig. 2); the maternal heart rates were 220–280/min. The anaesthetic and experimental procedures probably obscured any direct relationship between the time of gestation and foetal heart rate; however, in any one foetus under observation the heart rate was steady, declining about 10–20 beats/min during an hour's experiment. Incision of the uterine wall and exposure of the foetus depressed the heart rate by 10–20 beats/min.

During the administration of 10 % O_2 to the mother for a 5-min period, a slight foetal bradycardia was observed; with the administration of 6 % O_2 a reduction in foetal heart rate to 80–100 beats/min occurred within 3 min (Fig. 3). The response of the foetal heart to these low-oxygen mixtures was the same in eleven guinea-pigs from the fortieth day of pregnancy to term.

The foetal bradycardia observed in the following experiments was independent of the integrity of the vagus and represented the response of the pace-maker to asphyxia. After the administration of vasoconstrictor

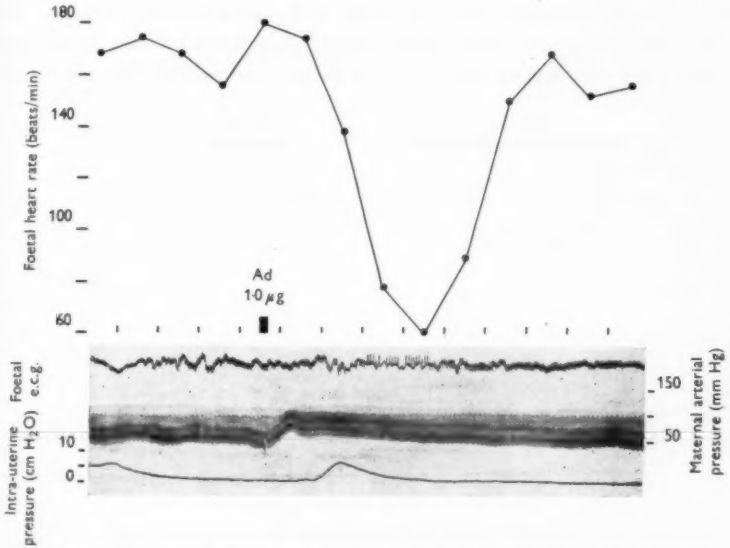

Fig. 1. To show that in the guinea-pig near term foetal bradycardia, following the maternal injection of 1·0 μg adrenaline, occurs after 30–45 sec during the decline of the maternal blood pressure to the resting level. Time marker, 10 sec.

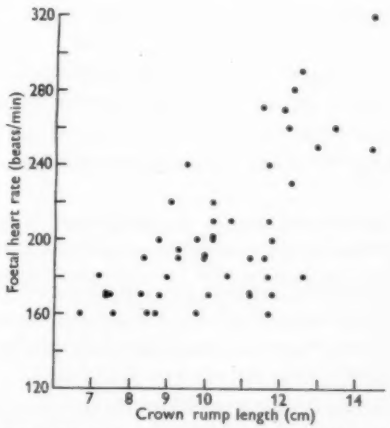

Fig. 2. The relationship between crown–rump length and heart rate of guinea-pig foetuses *in utero*.

substances to the mother, the latent period of the onset of the bradycardia was the same, 30–45 sec, (i) with the uterus intact, (ii) after incision of the uterine wall, and (iii) after cutting both vagi of the foetus in the neck. The situation was not so clear-cut in the older foetuses. The latent period of the bradycardia was the same, but occasionally it was preceded, or more often followed, by a tachycardia. A similar cardiac acceleration was also observed during the recovery from the maternal administration

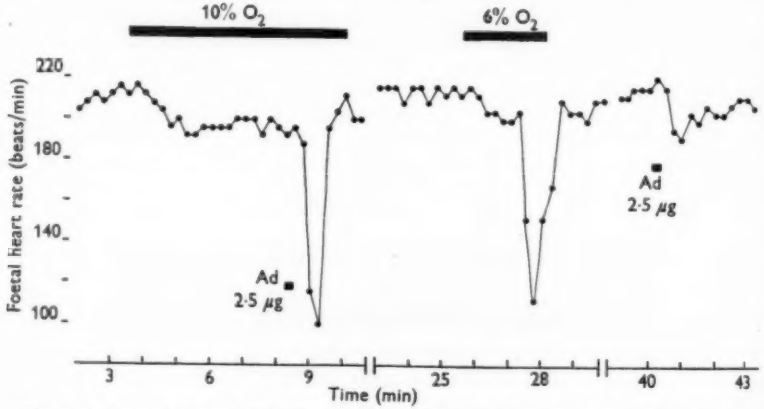

Fig. 3. Guinea-pig foetus, 60 days: to demonstrate that the maternal administration (i) of 10% oxygen causes a drop in foetal heart rate of 20 beats/min; (ii) of 6% oxygen rapidly causes foetal bradycardia to 100 beats/min. During the administration of 10% oxygen to the mother, foetal bradycardia is more readily obtained with 2·5 μg adrenaline. This pattern of response to low oxygen tensions is obtained from 40 days to term in the guinea-pig and it is concluded that the sensitivity of the foetal heart to hypoxia does not alter during this period.

of 6% oxygen. Such increases in heart rate are possibly due to a transient stimulation of the foetal cardioaccelerator centre or suprarenal medulla by the reduced tissue oxygen tension. Paul & Reynolds (1958) have quoted similar observations in the sheep foetus near term.

The influence of gestational age on the occurrence of foetal bradycardia following the injection of vasoconstrictors into the maternal circulation

At the end of the gestation period, from the sixtieth day of pregnancy to term (crown–rump lengths over 10 cm) 1·0–2·5 μg adrenaline injected into the maternal circulation was the smallest dose which would produce a transient foetal bradycardia, to 80–100 beats/min; the mean maternal arterial blood pressure rose 25–50 mm Hg, there was visible constriction of the uterine arteries and blanching of the uterine wall, and the foetal bradycardia occurred 30–45 sec after the injection, during the decline of the

maternal blood pressure to its resting level (Fig. 1). Accompanying these changes were intermittent movements of the uterine wall and occasionally a fall of 1 cm H_2O from the resting intra-amniotic pressure of 5 cm H_2O.

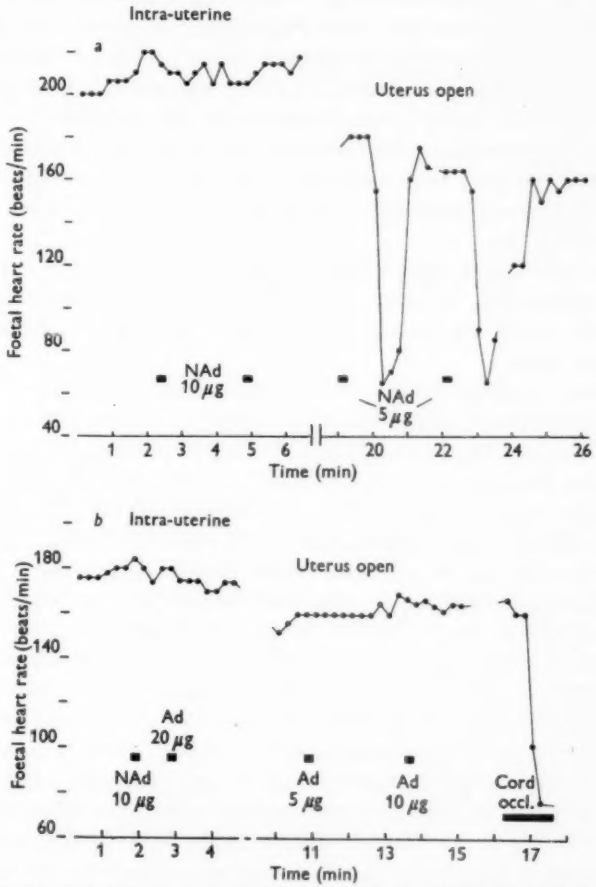

Fig. 4. To show that, in the guinea-pig, the sensitivity of the maternal placental vessels to vasoconstrictor drugs increases as gestation advances. (*a*) 5 μg noradrenaline in the maternal circulation causes foetal bradycardia only after the uterine wall has been opened at 54 days gestation. (*b*) 10 μg adrenaline in the maternal circulation does not cause foetal bradycardia even after the uterine wall has been opened at 47 days gestation; but cord occlusion causes prompt bradycardia.

From 50 to 60 days gestation (crown–rump lengths 8–10 cm) 2·5 μg adrenaline caused foetal bradycardia only after the uterine wall had been opened (Fig. 4*a*) and in still younger foetuses 5–10 μg adrenaline, though

causing a rise in maternal arterial pressure of 60 mm Hg, did not cause foetal bradycardia (Fig. 4*b*). Neither at these earlier stages of gestation was there any visible reduction in size of the uterine arteries nor blanching of the uterine wall: occasionally there was a fall in intra-amniotic pressure. Eleven pregnant guinea-pigs were studied in the gestational age group 40–60 days, and sixteen from the sixtieth day of pregnancy to term. Other vasoconstrictor drugs, noradrenaline (2·5 μg/kg), serotonin (50 μg/kg) and ergometrine (25–50 μg/kg), when injected into the maternal blood stream, also caused bradycardia of the foetus *in utero* at the end of gestation only; there was a marked tachyphylaxis after serotonin. Vasopressin (10–100 m-u./kg) did not cause foetal bradycardia at any time of the gestation period studied.

Sensory nerve stimulation was carried out to produce stress in the pregnant guinea-pig. In spite of the general excitation caused there was rarely even a small rise in the maternal arterial pressure or reduction of foetal heart rate.

The foetal bradycardia observed *in utero* at the end of gestation, after the administration of any of the vasoconstrictor drugs, was abolished by dibenzyline (5 mg/kg, Fig. 5*a*), which also prevented the spontaneous intermittent bradycardia which occurred in some experiments (Fig. 5*b*). Occasionally there was a transient acceleration of the foetal heart rate following the injection of adrenaline, which possibly crossed the placental barrier when the maternal vessels were unable to contract; adrenaline causes foetal tachycardia, following the injection into the vitelline vessels of amounts which are equivalent, weight for weight, with those causing tachycardia in the adult. In the presence of dibenzyline the maternal arterial pressure did not respond to the injection of adrenaline, noradrenaline, serotonin, or ergometrine, and there was no reduction in calibre of the uterine arteries or blanching of the uterus; the responses of the uterine muscle to these drugs and to oxytocin were unaltered. The absence of a fall in arterial pressure with adrenaline after dibenzyline suggests that, as in the rabbit (Nickerson, 1949), this drug produces no dilator action in the guinea-pig; Nickerson also observed that dibenzyline did not influence uterine responses.

The occurrence of foetal bradycardia following the maternal injection of oxytocin

Oxytocin 50 m-u./kg, although causing activity of the uterine muscle and intra-amniotic pressure swings of 5 cm H_2O, did not cause slowing of the foetal heart at any gestational age (Fig. 6); larger doses caused greater activity of the uterine muscle, with intra-amniotic pressure swings of 10–15 cm H_2O and frequently an intermittent slowing of the foetal heart,

which might or might not be in phase with the rise in intra-amniotic pressure. Syntocinon was used in twelve experiments and Pitocin in five.

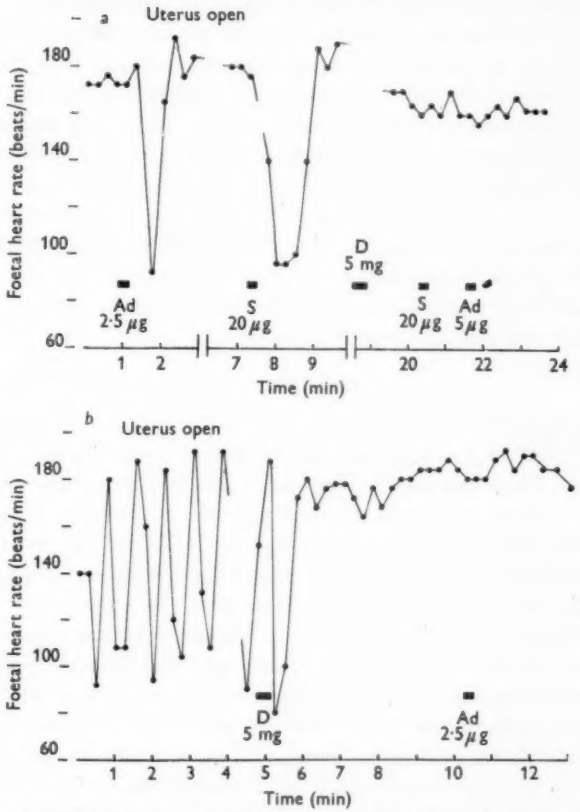

Fig. 5. Dibenzyline (D) prevents foetal bradycardia in the guinea-pig near term (*a*) following the injection of vasoconstrictor substances (serotonin, S) into the maternal circulation or (*b*) that arising spontaneously.

The influence of oestrogen and progesterone, at different gestation ages, on the occurrence of foetal bradycardia following the injection of adrenaline into the maternal circulation

The sensitivity of the uterine vessels to vasoconstrictor substances was determined by finding the smallest dose which, after injection into the maternal circulation, would cause a transient foetal bradycardia to 80–100 beats/min. The findings after the injection of hormones were compared with those discussed above, in which the effect of gestational age alone was studied.

Large doses of oestrogen are known to terminate pregnancy in a number of species, and in the present series of experiments three out of fourteen litters were dead 24 hr after the administration of the hormone. Oestrogen sensitized the maternal placental vessels to adrenaline, in the eleven animals studied at gestational ages from 40 days to term, 0·1–0·25 μg causing a transient foetal bradycardia. Progesterone also increased the sensitivity of the uterine vessels to adrenaline, but at the end of gestation only, from the sixtieth day of pregnancy to term; Fig. 7 shows the bradycardia in a 64-day-old foetus following the maternal injection of 0·25 μg adrenaline. Five animals were studied between the gestational age of 40 and 60 days and five from the sixtieth day of pregnancy to term. There were eleven and eighteen guinea-pigs in the corresponding control groups; in two of the latter group the uterine arteries were as sensitive to adrenaline as after hormone administration.

DISCUSSION

In these experiments the response of the uterine vessels to vasoconstrictor substances has been studied, and therefore the uterine blood flow should have been measured. However, this is difficult and an indirect method has been employed to demonstrate a decrease in blood flow, namely asphyxial slowing of the foetal heart; there are no observations which correlate changes in uterine blood flow with the degree of foetal bradycardia, but a gradual fall in foetal heart rate occurs as the maternal arterial oxygen tension is progressively lowered. In our experiments the rate fell to 80–100 beats/min, the same rate as that produced by total occlusion of the uterine arteries or the umbilical cord. It must be assumed, therefore, that the uterine blood flow was temporarily severely reduced by the vasoconstrictor substances studied. This is probably the reason why adrenaline is not transferred across the placental barrier from the mother to the foetus, though the evidence is indirect and not derived from estimations of foetal blood levels.

The present results confirm the previous observations that the transient foetal bradycardia following the maternal injection of vasoconstrictor substances is due to asphyxia consequent upon a reduction in maternal placental blood flow (Dornhorst & Young, 1952), for no increase in intra-amniotic pressure was ever observed and the maternal administration of the adrenergic blocking substance dibenzyline prevented the foetal bradycardia. The local movements of the uterine wall were also absent in the presence of dibenzyline and it is probable that these local contractions were induced by the asphyxia resulting from the reduction in uterine blood flow. Vasopressin did not cause foetal bradycardia in the guinea-pig, though Bengtsson (Smyth, 1958) has observed intraplacental

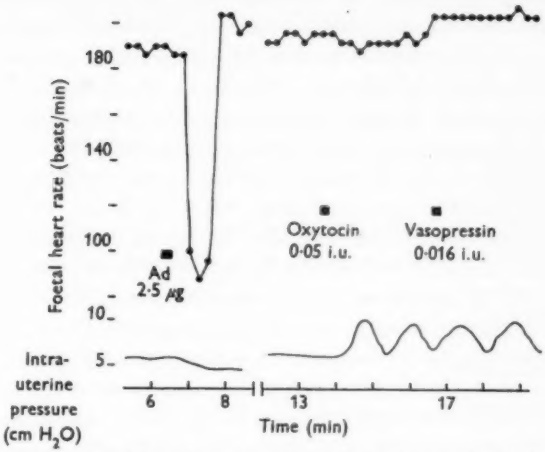

Fig. 6. Oxytocin injected into the maternal circulation in a moderate dose did not alter the foetal heart rate (guinea-pig 64 days); the uterine contractions caused fluctuations of 5 mm Hg in the intra-amniotic pressure.

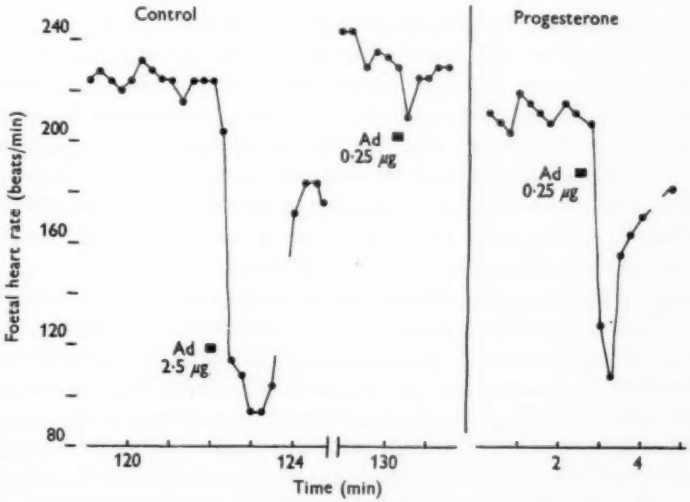

Fig. 7. Progesterone sensitizes the uterine vessels to adrenaline at the end of gestation in the guinea-pig. 10 mg progesterone was given 24 hr before the experiment, and 0·25 μg adrenaline in the maternal circulation caused foetal bradycardia to 80 beats/min; in the control animal of the same age 2·5 μg adrenaline was needed to cause equivalent bradycardia.

haemorrhages in the rabbit following the maternal injection of larger doses of vasopressin; he also recorded a slowing of the foetal heart rate in non-viable human foetuses after the administration of vasopressin to the mother.

Reynolds & Paul (1955) observed foetal bradycardia in the sheep following the injection of large doses of oxytocin causing similar changes in intra-amniotic pressure as those observed in the present experiments. Assali, Dasgupta, Kolin & Holms (1958) have recorded a decrease in uterine blood flow in the pregnant dog, following the injection of oxytocin. It has also been observed that when the foetus is exposed the uterine muscle contracts away from the incision towards its mesenteric border, round the entry of the blood vessels into the uterus, and probably causes a reduction in uterine blood flow and foetal asphyxia. This is suggested by the observations that (i) the control heart rate is usually 10–20 beats/min slower than when the uterine wall is intact and (ii) that slowing of the foetal heart to 80–100 beats/min can be produced with smaller doses of adrenaline in the maternal circulation than when the foetus is *in utero*.

Sensitivity of the foetal heart to hypoxia during gestation

Greenfield & Shepherd (1953) have also observed in the guinea-pig that the administration of low-oxygen mixtures to the mother caused the same pattern of changes in the foetal heart rate at all stages of gestation. The readiness with which, at the end of the gestation period, foetal bradycardia occurs following the administration to the mother of vasoconstrictor drugs is therefore not due to an increased sensitivity of the foetal heart to hypoxia. This is in keeping with the findings (i) that foetal oxygen consumption is about 5 ml./kg/min throughout gestation (Acheson, Dawes & Mott, 1957); Assali, Rauramo & Peltonen, 1960); and (ii) that as the umbilical blood flow increases with gestational age, the volume flow which lays down a unit weight of foetal tissue remains constant throughout intra-uterine life (Greenfield, Shepherd & Whelan, 1951).

A slight reduction in foetal heart rate was observed during the inhalation of O_2 10% in nitrogen by the mother, and assuming that there was only a small alteration in maternal placental blood flow it might be predicted that the foetal oxygen supply was approximately halved. It is, therefore, suggested that no slowing of the foetal heart was observed, following the intravenous injection of vasoconstrictor substances into the mother, until the maternal placental blood flow had been reduced by at least 50%. During the administration of O_2 10% to the mother slowing of the foetal heart to 80–100 beats/min can be produced by smaller doses of adrenaline into the maternal circulation than while she is breathing air; a smaller reduction in maternal placental blood flow and therefore of oxygen supply is required to bring about bradycardia in a foetus which is already hypoxic.

During the administration of oxygen 6% in nitrogen to the mother the foetal heart rate dropped suddenly to 80–100 beats/min, the lowest level observed during the transient bradycardia in response to the maternal administration of vasoconstrictor drugs. This heart rate possibly represents the value characteristic of the activity of the pace-maker during short periods under practically anaerobic conditions, for it is the rate observed when the umbilical cord is clamped.

The influence of the ovarian hormones on the sensitivity of the maternal placental vessels to vasoconstrictor drugs

As the ovarian hormones control the vascularity of the reproductive organs, it is reasonable to suppose that they might also influence the response of the placental vessels to various substances, including other hormones. Since there is no information about the blood concentration and excretion rates of oestrogenic and luteal hormones during pregnancy in the guinea-pig, the following discussion is only speculative.

It is known that the plasma concentration of progesterone increases at the end of pregnancy in man (Short & Eton, 1959) and it is possible that a rise in circulating progesterone may be responsible for the increased sensitivity of the maternal placental vessels to vasoconstrictor drugs observed at the end of pregnancy in the guinea-pig. Indirect support for this hypothesis comes from the experimental finding that the administration of additional progesterone to the mother at this time enhances still further the activity of vasoconstrictor drugs on the maternal placental vessels. The mode of action of progesterone under these circumstances may be similar to that suggested by Lavollay & Neumann (1949) for the potentiating influence of progesterone on adrenaline in reducing capillary permeability; the rate of auto-oxidation of adrenaline is decreased by progesterone, and ascorbic acid, tochopherol and adrenocorticotrophic hormone each have a similar effect. The electrolyte changes brought about by progesterone may also play a part in altering the response of the vessels to vasoconstrictor substances (Vick, Ederstrom & Vergeer, 1956).

The sensitization of the maternal blood vessels by progesterone and by oestrogen cannot be general in the guinea-pig, for the arterial blood-pressure responses remained normal. Mastboom & Horsten (1956) obtained similar results in the rabbit, but Lloyd (1958) observed systemic changes in sensitivity as well as local changes in the mesenteric vessels of the rat with both progesterone and oestrogen.

Possible clinical significance of results

Foetal bradycardia which cannot be explained by mechanical changes within the uterus is frequently observed in the full-time infant, and the

results reported in the guinea-pig suggest that a reduction in maternal placental blood flow, due to hormonal influences, is most likely to occur as term approaches. There is some evidence for an increased production of sympathetic amines during difficult labour: Garcia & Garcia (1955) showed that 'epinephrine-like substances' are readily demonstrable in patients' plasma during painful and inert labours, and Gemzell, Robbe & Ström (1956) found an increased excretion of catechol amines in two cases with post-partum haemorrhage; normal labour did not influence either the blood levels or the excretion rates of sympathetic amines. If an increased production of vasoconstrictor substance by the mother during difficult labour proves to be the cause of the reduction in maternal placental flow, then dibenzyline may provide one means of preventing this and its consequences for the infant.

If one of the causes of toxaemia of pregnancy proves to be an unbalance in the production of oestrogenic and luteal hormones, as suggested by Smith & Smith (1938), then these authors' conception of this unbalance as a cause of vascular derangement, and particularly of an increased sensitivity of the maternal placental vessels to vasoconstrictor substances, would have some support. In both the present work on guinea-pigs and that of Lloyd (1958) on the rat excess of either hormone appears to sensitize the maternal blood vessels to constrictor drugs.

SUMMARY

1. A transient foetal bradycardia, following the injection into the maternal circulation of a vasoconstrictor substance, was obtained in the guinea-pig only at the end of the gestation period, from the sixtieth day of pregnancy to term (67 days). Adrenaline, noradrenaline, serotonin and ergometrine were effective, vasopressin was not.

2. The foetal bradycardia was due to the reduction in maternal placental blood flow, with consequent intra-uterine asphyxia; it was prevented by dibenzyline.

3. The readiness with which foetal bradycardia can be produced at the end of pregnancy is not due to the increased sensitivity of the foetal heart to low oxygen tensions, for the same pattern of response following the administration of low-oxygen mixtures to the mother was obtained at all stages in the gestation period studied, from the fortieth day of pregnancy to term.

4. The maternal uterine and placental vessels are sensitized hormonally to the influence of vasoconstrictor substances at the end of the gestation period. Oestrogen administration to the mother increased the sensitivity of the maternal uterine and placental vessels from the fortieth day, and

progesterone administration from the sixtieth day of the pregnancy; there was no concurrent alteration in the response of the systemic arterial blood pressure to vasoconstrictor drugs.

5. Foetal bradycardia also occurs following the maternal injection of *large* doses of oxytocin, which increase intra-uterine pressure.

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THE OUTPUT OF ADRENALINE AND NORADRENALINE FROM THE ADRENAL MEDULLA OF THE CALF

By MARIAN SILVER
From the Physiological Laboratory, University of Cambridge

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The adrenal medulla contains both adrenaline and noradrenaline but their proportions vary considerably in different species: from 99–100% adrenaline in the rabbit to 20–30% in the whale (for references see von Euler, 1956). In the cat and dog, stimulation of the glands leads to the liberation of adrenaline and noradrenaline in approximately the same proportions as in the medulla (Lund, 1951; Outschoorn, 1952; Vogt, 1952; Rapela, 1956; Butterworth & Mann, 1957), but it is not certain whether a similar parallel between output and content occurs in other species.

In contrast with the wide variation in the relative and absolute pressor amine content of adult adrenal glands, foetal adrenal medullary tissue contains primarily noradrenaline (Shepherd & West, 1951; West, Shepherd, Hunter & MacGregor, 1953). There is a gradual increase in the percentage of adrenaline with age, but the adult stage may not be reached until some months or even years after birth (Holton, 1951; Shepherd & West, 1951; Hökfelt, 1952; West *et al.* 1953). It seemed of interest to study the output of pressor amines from the adrenal glands of young animals of various ages, and to determine whether the change in the proportion of adrenaline in the glands was accompanied by any similar changes in output. Preliminary experiments on the lamb (Comline & Silver, 1958) showed that, in this species, the major increase in the proportion of adrenaline in the adrenal glands occurred *in utero*. During the latter part of pre-natal life there was an increase in the proportion of adrenaline released during splanchnic nerve stimulation, which paralleled the rise in the percentage of this amine in the glands. The present work is concerned with similar experiments on the post-natal development of adrenal medullary function in the calf, a preliminary account of which has already been published (Silver, 1954).

METHODS

Male Jersey calves from 3 hr to 220 days, and adult cows of the same breed aged 2–3 years, were used in these experiments. Food was withheld from the young animals on a milk diet on the morning of the experiment; from weaned animals, solid food, but not water, was withdrawn for 36–48 hr before the experiment.

Anaesthesia was induced in the calves by ethyl chloride and ether and thereafter maintained by intravenous chloralose (50 mg/kg). The adult cows were anaesthetized by the intravenous injection of Na pentobarbitone (10 mg/kg).

The left adrenal gland was prepared for the collection of adrenal effluent blood. It was exposed by a flank incision, and in the young calf its dissection was facilitated by removal of the last rib. In all animals the left kidney was removed, and after cannulation of the renal vein, blood from the adrenal gland was diverted backwards into the cannula by means of a Blaylock clamp placed across the junction of the renal vein with vena cava, just medial to the entrance of the very short adrenal vein. Coagulation of the blood was prevented by intravenous heparin (1000 i.u./kg.). The adrenal effluent blood was returned to the general circulation through an external circuit of polythene tubing connected via a drop chamber to the femoral vein. The left splanchnic nerve was exposed and cut as close as possible to its point of emergence through the diaphragm, thereby leaving 3–4 cm of the peripheral end free for stimulation. In all experiments blood pressure was recorded from the carotid artery to check the efficacy of splanchnic nerve stimulation.

Adrenal effluent blood. Samples of about 20 ml. were collected through a side arm inserted as close to the renal-vein cannula as possible. The sampling time varied from a half to 1 min in the cows and from ¼ to 2 min in the calves, depending on the rate of blood flow, and there was no evidence that pressor amine output changed during this time; in any one animal the sampling period was kept constant. A resting sample of adrenal blood was taken at the beginning of each experiment about 30 min after the left splanchnic nerve had been cut; subsequent samples were taken during periods of stimulation starting 15–30 sec after the onset to allow for the dead space. When the splanchnic nerve was stimulated continuously for 40 min, blood samples were collected at intervals during this period. Siliconed glassware was used throughout, and after collection the blood was immediately centrifuged on ice.

Stimulation of the peripheral end of the left splanchnic nerve with platinum electrodes was carried out either from a Palmer induction coil (frequency *ca.*/80 sec) or by condenser discharges at either, 5, 10 or 30/sec; the stimulus strength was always adjusted to give a maximal increase in blood pressure on splanchnic nerve stimulation. No special precautions were taken to prevent polarization of the electrodes or deterioration of the nerve, but neither of these factors appeared to be affecting the response, since the frequent removal of the electrodes from the nerve for a few seconds (ensuring depolarization) and changing their position along the length of the nerve did not cause any increase in the blood-pressure response.

Acetylcholine (1–3 mg) was given by close arterial injection into the anterior mesenteric artery. The coeliac artery was tied previously and the aorta occluded caudal to the left adrenal gland during the period of injection. Administration of atropine sulphate (0·2 mg/kg i.v.) before each acetylcholine test prevented all cardiovascular effects of any acetylcholine reaching the general circulation. The dose of acetylcholine varied with the age of the animal: 1 mg induced a maximal response in calves under 1 week of age, 3 mg was given to calves of 1–3 months.

Adrenal glands. The adrenaline and noradrenaline content of the adrenal glands of calves of different ages was determined from the right gland, which was removed at the end of each experiment. In those animals subjected to prolonged splanchnic nerve stimulation both glands were removed immediately after the end of stimulation. No glands were used for pressor amine estimations after acetylcholine injections.

Adrenaline and noradrenaline were separated chromatographically before estimation, by the method of Crawford & Outschoorn (1951) as modified by Vogt (1952). 5–7 ml. samples of plasma were used for extraction, and after the final evaporation of the eluates from the chromatograms the amines were taken up in 1 ml. NaCl 0·9 % (w/v). The adrenal glands were ground with sand and 0·1 N-HCl, 10 ml. acid/g tissue; 1 ml. samples of the homogenate were used for extraction. In the majority of experiments the final eluates from plasma and

adrenal tissue were assayed biologically, either by the blood-pressure response of the anaesthetized rat given atropine and hexamethonium to increase the sensitivity (Outschoorn, 1952), or by the inhibition of carbachol-induced contractions of the isolated rat uterus (Gaddum & Lembeck, 1949). In general four-point assays were carried out on each sample. L-Adrenaline-HCl or bitartrate and L-noradrenaline bitartrate were used for the preparation of standard solutions and the quantities are expressed as weights of base. No systematic search for other pressor amines or their derivatives was made, but several chromatograms of plasma and adrenal tissue were analysed by assaying the eluates from paper strips taken at levels other than those corresponding to adrenaline and noradrenaline; no pressor activity was found in these samples.

Recently the fluorimetric method of von Euler & Floding (1955) has also been employed for catechol estimation, after chromatographic separation and elution of the amines. When compared with biological assay in a series of analyses of adrenaline and noradrenaline in plasma, with twelve random samples of each, there was no consistent difference between the results obtained by the two methods of estimation. Mean figures for each series were (μg/ml.): noradrenaline 1·16 (assay and fluorimetry), mean difference $0{\cdot}005 \pm 0{\cdot}06$; adrenaline, 1·30 (assay) and 1·31 (fluorimetry), mean difference $0{\cdot}01 \pm 0{\cdot}03$. From this series of analyses the fluorimetric method appears to be unbiased; a detailed statistical investigation into the comparative reliability of fluorimetry and biological assay for the estimation of pressor amines will be presented elsewhere.

RESULTS

Adrenaline and noradrenaline in the adrenal glands

Preliminary experiments showed that the adrenaline and noradrenaline concentrations of the right and left unstimulated adrenal glands of the calf were indistinguishable. The right adrenal, which remained intact during the course of these experiments, was therefore used to examine the changes in content and proportions of the two amines at different ages. It was assumed that the right gland would be unaffected by stimulation of the left splanchnic nerve, and that the slight depletion due to endogenous splanchnic discharge during the course of the experiment (Elmes & Jefferson, 1942) would be small compared with the high amine concentration in the medulla.

The results given in Table 1 show that there is an increase in the concentration of adrenaline and a fall in that of noradrenaline during the 2nd week of life with a consequent increase in the proportion of adrenaline during this period. However, when the results are expressed as total amounts of amines per adrenal gland (Fig. 1), it is seen that the noradrenaline content remains constant throughout the period investigated, whereas the adrenaline content rises from the second week onwards. The discrepancy between the figures for pressor amine concentration (Table 1) and those for total glandular content (Fig. 1), can presumably be ascribed to the more rapid growth of the adrenal cortex relative to the medulla in the growing calf.

TABLE 1. Change in adrenaline and noradrenaline concentration in the adrenal glands with age

Age	Number of animals	Mean adrenal weight (g)	Mean pressor amine concentration (mg/g adrenal tissue)		Mean % adrenaline ±S.E.	
			Noradrenaline	Adrenaline		
Calf (weeks)						
Birth–1	10	1·6	0·86	0·91	51 ± 1·7	$P < 0·01$
2–3	6	1·8	0·53	1·11	67 ± 2·4	
4–5	3	1·9	0·65	1·54	71	
11	2	2·4	0·67	1·5	69	
15–18	4	3·7	0·5	1·49	75	
Cow (2–3 yr)						
Non-pregnant	4	9·9	0·42	1·22	75	
Pregnant	3	12·2	0·29	1·07	79	

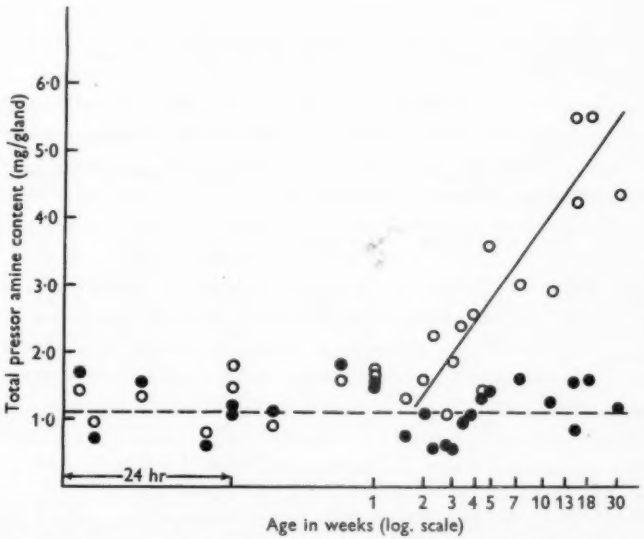

Fig. 1. The change in the total amount of adrenaline and noradrenaline in the adrenal glands of the calf with age; logarithmic time scale. ●, mg noradrenaline/gland; ○, mg adrenaline/gland; ———, regression line fitted by the method of least squares from data from animals of over 10 days of age; dependent variable, glandular content of adrenaline (regression coefficient $= 3·59 \pm 0·59$, $P < 0·001$).

Adrenaline and noradrenaline in the adrenal effluent blood

Resting level of secretion from acutely denervated gland

This is summarized in Table 2. The amounts secreted were always small and the proportion of adrenaline to noradrenaline in the effluent blood

corresponded approximately to that found in the gland, the percentage of adrenaline increasing after the first week of life. When expressed as μg/kg body weight/min the total resting output in the older calves was comparable with that found by Dunér (1953) and Rapela (1956) using the acutely denervated adrenal glands of the cat and dog, respectively.

TABLE 2. Resting level of secretion from calf adrenal medulla: splanchnic nerve cut ½ hr previously

Age (weeks)	Number of animals	Mean rate of secretion of pressor amines into adrenal effluent blood: Noradrenaline (μg/min)	Adrenaline (μg/min)	Total output (μg/kg/min)	Mean % adrenaline ± S.E.	
Birth–1	8	0·65	0·55	0·047	44 ± 6·1	0·05 > P > 0·02
2–5	8	0·24	0·45	0·027	66 ± 7·5	
8–30	8	0·23	0·75	0·017	75 ± 7·5	P > 0·1

TABLE 3. Rate of output of pressor amines from the adrenal medulla during ½–2 min stimulation of the peripheral end of the splanchnic nerve

Age	No. of animals	Mean rate of pressor amine output (μg/min ± S.E.): Noradrenaline	Adrenaline	(μg/kg body wt./min): Noradrenaline	Adrenaline	Mean % adrenaline: Effluent blood	Right gland
Calf							
1st 24 hr	5	1·2 ± 0·25	3·2 ± 0·80	0·07	0·15	73	51
½–1 week	13	7·0 ± 1·3	5·0 ± 1·0	0·27	0·20	42	51
2–3 weeks	8	15 ± 3·6	8·1 ± 1·2	0·67	0·39	36	66
4–5 weeks	7	17 ± 2·2	11 ± 0·54	0·60	0·43	41	71
7 weeks	4	18 ± 5·8	13 ± 2·0	0·63	0·45	42	—
11 weeks	3	30	29	0·66	0·61	49	69
15–18 weeks	5	32 ± 8·8	39 ± 8·5	0·42	0·55	54	75
30 weeks	4	31 ± 7·3	39 ± 3·9	0·30	0·48	56	—
Cow 2–3 yr							
Non-pregnant	3	23	60	0·055	0·14	72	75
Pregnant	5	17 ± 4·7	45 ± 9·0	0·045	0·13	73	79

Level of secretion during splanchnic nerve stimulation. Short period of stimulation (½–2 min)

Induction coil (*ca.* 80/*sec*). The mean rates of pressor amine secretion from the adrenal medulla during splanchnic nerve stimulation in calves of different ages and in the adult are given in Table 3. The rate of output of both amines increased with age, but this rise in the level of adrenal medullary secretion was not directly related to general body growth, since the major increases in pressor amine output occurred during the first few weeks of life, at a time when changes in body weight are negligible. Hence an even larger initial increase in adrenaline and noradrenaline output is found when the data are calculated as μg/kg body weight/min (Table 3).

Noradrenaline output was higher than that of adrenaline in calves from $\frac{1}{2}$ to 7 weeks of life, while after 15–18 weeks the same type of stimulus released larger amounts of adrenaline. Comparison of individual observations showed that both these differences were statistically significant ($P = 0{\cdot}01$).

In the adult the rate of noradrenaline secretion was considerably lower, and that of adrenaline higher, than in calves of 15–30 weeks. The differences between pregnant and non-pregnant animals (Table 3) were negligible when the data were calculated as μg/kg body wt./min.

The contrast between adrenal medullary secretion in the calf and in the adult is emphasized when the figures for percentage adrenaline in the effluent blood are compared with those for the glands (Table 3). In the adult the two values are almost equal, while in all calves tested after 24 hr from birth the percentage of adrenaline in the effluent was much lower than that in the gland. This very striking difference between the proportions of the two amines released and their proportions in the gland seemed to be due almost entirely to the unexpected increase in the rate of noradrenaline output with age, which was difficult to reconcile with the constant total amount of this amine in the glands throughout the period investigated (Fig. 1). Further experiments were therefore carried out to study the factors concerned in the high noradrenaline output in the calf, and to determine whether such a level of secretion could be maintained for long periods of stimulation.

Comparison between stimulation at ca. 80/sec and at lower rates. The rate of stimulation employed in the preceding experiments was *ca.* 80/sec, and since the probable maximum rate of endogenous splanchnic nerve discharge is considerably lower than this (Celander, 1954; Rapela, 1956), the secretions obtained with three low frequencies of stimulation (5, 10 and 30/sec) were also studied. The different frequencies of stimulation were applied in random order; the rest period between successive tests was 15–20 min. Four calves of 3–4 weeks old were used for this series since it was at this age that the divergence between glandular content and output was greatest.

The proportions of the two amines released by nervous stimulation were not affected by the rate at which the splanchnic nerve was made to discharge (Fig. 2). However, the rate of both adrenaline and noradrenaline output rose with frequency of stimulation to a maximum at 30 stimuli/sec. Rapela (1956) also found that in the adult dog stimulation at 40/sec gave a bigger response than either 10/sec or 160/sec, but he reports a significant increase in the percentage of noradrenaline.

Comparison between the effect of acetylcholine and nerve stimulation. The mean levels of adrenaline and noradrenaline secreted in response to

acetylcholine in four calves aged 3–4 weeks were compared with the results obtained with different rates of nerve stimulation. It will be seen from Fig. 2 that acetylcholine resulted in the output of a very much larger amount of noradrenaline than any frequency of nerve stimulation. The proportion of adrenaline in the effluent blood after acetylcholine was only 22%, compared with 40–44% during splanchnic stimulation and 70% in the glands.

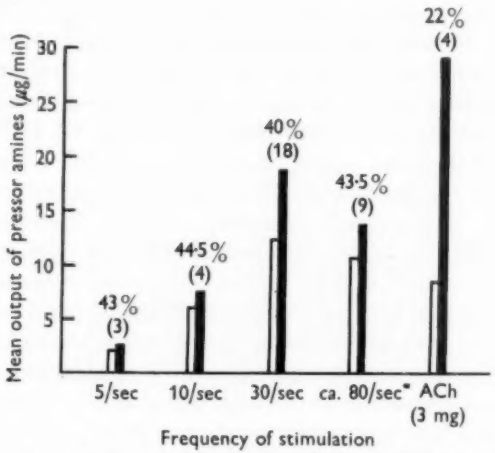

Fig. 2 Mean output of pressor amines from the adrenal glands of calves aged 3–4 weeks, obtained during nerve stimulation with different frequencies, and after the intra-arterial injection of acetylcholine: □ adrenaline; ■, noradrenaline; mean % adrenaline given above each histogram; number of observations in parenthesis. * Induction coil.

Prolonged stimulation (induction coil)

Continuous stimulation. The course of adrenal medullary secretion during 40 min continuous splanchnic nerve stimulation was followed in calves of different ages and in three adults. The mean values for adrenaline and noradrenaline output in each age-group are plotted against duration of stimulation in Fig. 3: the changes in the proportion of adrenaline in the effluent blood are shown in the upper part of each graph. In the first few days of life (Fig. 3*a*) the initial output of both amines was very low and showed no marked changes during the period of prolonged stimulation. In all other groups of calves and in the adult animals the levels of both adrenaline and noradrenaline decreased with continuous splanchnic stimulation; the higher the initial rate of secretion, the greater was the fall in rate of output during the 40 min period. But whereas in the adult the output of both amines declined at the same rate, in the calves the output of noradrenaline fell more quickly. This fall was greatest in the

first 10 min of stimulation and after 40 min the output had dropped to one third of the initial level. In contrast, the adrenaline secretion changed little during the first 25 min of stimulation, but fell more quickly during the last 15 min (Fig. 3*b–e*). These changes are reflected in the proportion of adrenaline secreted during the stimulation period.

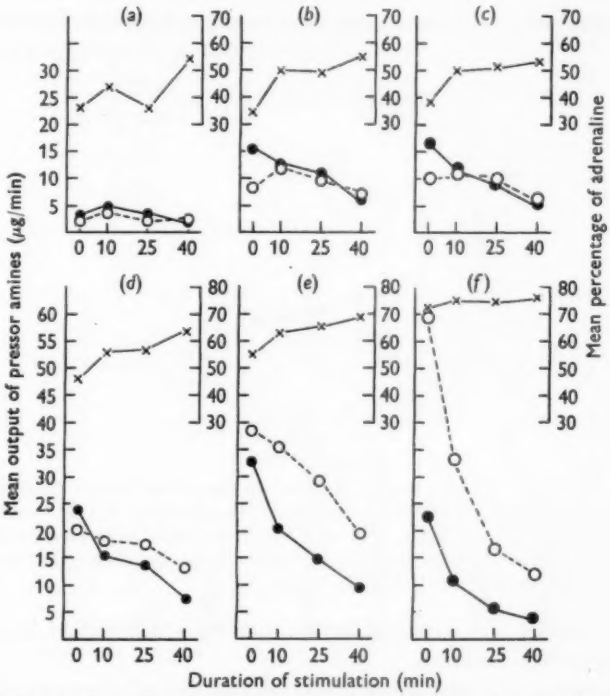

Fig. 3. Mean output of pressor amines from the bovine adrenal gland during prolonged splanchnic stimulation (induction coil). ●, noradrenaline; ○, adrenaline; ×, % adrenaline. Calves (*a*) under 1 week (9 animals); (*b*) 2–3 weeks (6 animals); (*c*) 4–5 weeks (5 animals); (*d*) 7–11 weeks (6 animals); (*e*) 15–30 weeks (9 animals). Cows (*f*) 2–3 years (3 animals).

In the adult the proportion of adrenaline in the effluent blood remained constant at 73–75 %, which is close to the corresponding values for adrenal glands. In the young animals the proportion of adrenaline rose by 13–20 %, the major increases in all groups occurring during the first 10 min of stimulation (Fig. 3*b–e*). Therefore, although the percentage of adrenaline in the blood initially was about 20–30 % lower than that in the glands, the final values after 40 min stimulation were only 5–10 % lower.

Intermittent stimulation. A similar change in the proportions of the two

amines in the adrenal effluent blood was found when an interval of 15–20 min occurred between brief periods of stimulation. The mean rates of adrenaline and noradrenaline secretion and the corresponding figures for the percentage of adrenaline, shown in Fig. 4, are similar to those obtained during continuous stimulation in calves of comparable age (Fig. 3*b*, *c*). The rise in the percentage of adrenaline which occurred with each successive stimulation was due primarily to a fall in noradrenaline output

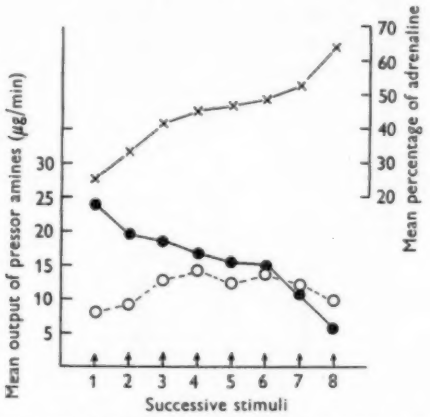

Fig. 4. The mean output of pressor amines from the adrenal glands of calves aged 3–4 weeks, during intermittent stimulation of the splanchnic nerve. Duration of stimulation ¾–2 min applied at 15–20 min intervals; rate of stimulation = 30 and *ca.* 80/sec, applied in random order. Each point represents a mean value for three or more observations. Symbols as for Fig. 3.

while the rate of adrenaline secretion, after a slight initial increase, remained relatively constant.

The amount of noradrenaline in the adrenal glands of the calves used in the preceding experiments was low compared with the adrenaline concentration (Fig. 1), and therefore it was important to determine whether lack of noradrenaline could be responsible for its rapid decline in output during prolonged stimulation. However, when the content of the left adrenal glands was investigated it was found that considerable amounts of both amines were still left after 40 min continuous stimulation; the mean level of noradrenaline was 0·98 mg/gland, while the adrenaline levels varied with age from 1·12 to 3·76 mg/gland.

Effect of acetylcholine given before and after a period of prolonged splanchnic nerve stimulation

The results given in the preceding section showed that the pressor amines in the adrenal medulla were by no means exhausted by 40 min

continuous stimulation of the splanchnic nerve, yet the output of both amines fell during this period. It was possible that not all the amines in the medulla were immediately available for release and that the decline in output reflected this. Alternatively a progressive failure in transmission would explain the changes in output. In the latter event direct excitation of the medulla with acetylcholine at the end of a period of prolonged nerve stimulation should restore the original secretory levels. Experiments were accordingly performed to test this possibility.

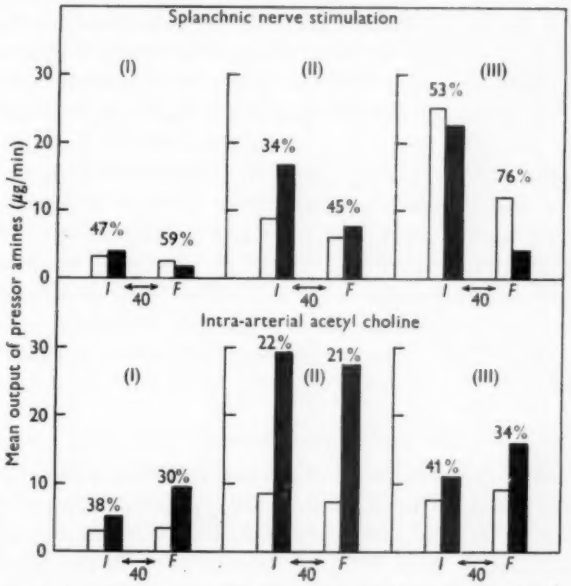

Fig. 5. Comparison between the effects of splanchnic nerve stimulation (above) and of intra-arterial injection of 1–3 mg acetylcholine (below), before and after a 40 min period of continuous nerve stimulation (←40→). *I*, mean initial output and *F*, final output of pressor amines; □, adrenaline; ■, noradrenaline. Mean % of adrenaline is given above each histogram: for further details see text.

The left adrenal gland was again subjected to 40 min continuous stimulation and the output of adrenaline and noradrenaline obtained at the beginning and end of this period was compared with the response elicited by the intra-arterial injection of acetylcholine given before and immediately after the stimulation period. Three groups of three calves, aged (i) under 1 week, (ii) 3–4 weeks, and (iii) 8–12 weeks, were used in these experiments. The mean levels of secretion obtained in each group are given in Fig. 5.

The results with prolonged splanchnic stimulation, shown in the upper

part of Fig. 5, are typical of those obtained in the previous experiments, with noradrenaline output falling to a greater extent than that of adrenaline. Acetylcholine, on the other hand, induced the secretion of large amounts of noradrenaline both before and after prolonged splanchnic stimulation. The high proportion of noradrenaline released initially by acetylcholine in all three groups confirmed the results obtained previously with 3–4 weeks old calves (see Fig. 2). However, in the present experiments, involving animals of widely differing ages, the absolute levels of noradrenaline secreted varied considerably; in groups (i) and (ii) acetylcholine was a far more effective stimulus for noradrenaline release than nerve stimulation, while in the oldest group of calves the response to acetylcholine was small compared with the effects of splanchnic discharge. Despite these variations in absolute levels, the percentage of adrenaline in the effluent blood after acetylcholine was considerably lower in all groups than that obtained with nerve stimulation. At the end of the 40 min period of continuous stimulation acetylcholine, in contrast to splanchnic nerve stimulation, released as much adrenaline and noradrenaline as at the beginning. These results indicate that in glands previously subjected to prolonged splanchnic discharge both adrenaline and noradrenaline were available for release and suggest that the fall in output obtained with continuous splanchnic stimulation was due to a gradual failure in transmission.

DISCUSSION

An increase in the percentage of adrenaline in the adrenal glands with age has been found in the calf, confirming previous reports on this and other species by Holton (1951), Shepherd & West (1951) and Hökfelt (1952). The increase, from 51 % at birth to 77 % in the adult, occurred early in post-natal life, and was brought about by a rise in the total amount of adrenaline in the glands while the noradrenaline content remained unchanged. The adrenal glands were not removed until the end of the experiment, and hence any losses due to endogenous splanchnic discharge during 2–3 hr anaesthesia could not be gauged. However, all studies on the pressor amine concentration of adrenal glands necessarily involve either anaesthesia or rapid killing before the glands can be removed, and the sympathetic discharge accompanying these procedures may well induce large adrenal medullary losses.

The development of adrenal medullary function in the calf, as judged by the response to splanchnic nerve stimulation, lagged behind the changes in content. Thus, immediately after birth the medulla was comparatively unresponsive to stimulation despite the presence of apparently adequate amounts of both amines in the glands. Nervous stimulation elicited a

response which was only 2–3 times the resting level of secretion, whereas a week later a twentyfold increase in output was obtained under similar conditions without any changes in glandular content.

In the older animals the proportion of noradrenaline in the adrenal effluent blood during stimulation was higher than that in the glands. This was a constant finding in calves from 1 to 30 weeks, and although the increase in glandular adrenaline with age was to some extent paralleled by a corresponding rise in adrenaline output, this relationship was obscured by the anomalous production of noradrenaline. In lambs no phase analogous to this was found either pre- or post-natally (Comline & Silver, 1958); the major increase in the percentage of adrenaline in the glands occurred in the foetus, but, in contrast to the calf, this change was closely paralleled by a rise in the proportion of adrenaline secreted on splanchnic stimulation.

In the few species in which adults have been studied a slightly greater proportion of noradrenaline is liberated on stimulation than is found in the gland, although some of the early work on the cat is at variance with this (see von Euler, 1956). In the cow this difference was only 4–6 % and was negligible compared with the discrepancy of 20–30 % in the calf. Nevertheless, the fact that a high proportion of noradrenaline has been found in the effluent blood of other species suggests that the phenomenon in the calf portrays a developmental phase which persists, to a limited extent, in adult life.

More detailed analysis of the response to splanchnic stimulation in calves showed that the characteristically high output of noradrenaline was obtained only during the initial periods of stimulation; the response rapidly waned with repeated or prolonged stimulation, while the amount of adrenaline released often increased and only fell during the last 15 min of prolonged stimulation. In the adult a fall in output of both amines occurred during continuous stimulation, but the percentage of adrenaline in the effluent blood remained unchanged. This finding in the cow agrees with the observations of Outschoorn (1952) on the adult cat.

The fall in pressor amine output with prolonged splanchnic stimulation could be attributed either to a progressive failure of transmission at the splanchnic nerve endings, or to a decrease in the concentration of available amines in the medulla, or to a combination of both these factors. The results with acetylcholine showed that there was no lack of releasable material at the end of the 40 min period of stimulation in the calf. In fact acetylcholine was a particularly effective stimulus, and it released an even greater proportion of noradrenaline than did splanchnic stimulation. This finding was not in accord with previous work on adult cats, in which both acetylcholine (Outschoorn, 1952; Butterworth & Mann, 1957) and KCl (Vogt,

1952) had the same effect as nerve stimulation, and released approximately the same proportion of adrenaline into the effluent blood as that found in the glands. In the calf, on the other hand, with only 30% noradrenaline in the glands as much as 80% noradrenaline was released after acetylcholine, but only 50–60% during nerve stimulation. These results suggested that the injected acetylcholine was not exciting the same cells as the acetylcholine released from the splanchnic nerve endings; for if the sites of action had been the same, then the proportions of the two amines released should have remained constant. The discrepancy between the effects of splanchnic stimulation and intra-arterial injections of acetylcholine can be explained, at least in part, if it is assumed that some of the noradrenaline-releasing cells in the medulla of immature animals are not directly excitable by nerve stimulation. These cells might, however, show a phenomenon akin to the sensitivity of denervation i.e. extreme sensitivity to acetylcholine until the tissue became fully innervated.

Histo-chemical evidence for separate adrenaline and noradrenaline secreting cells in the medulla has been provided by the work of Hillarp & Hökfelt (1954), and Eränkö (1955*a*, *b*), but there is as yet no direct histological evidence for their separate innervation, although the separate nervous control of adrenaline and noradrenaline release has been shown by hypothalamic stimulation (Redgate & Gelhorn, 1953; Folkow & von Euler, 1954). The rate of development of innervation in the immature adrenal gland is not known, although it may well be very slow; in human sympathetic ganglia development is not complete even at seven years after birth (Gairns & Garven, 1959).

The above hypothesis would go far to explain many of the results obtained in young calves, such as the preferential release of noradrenaline by acetylcholine, the discrepancy between content and initial output during nerve stimulation, and the smaller response to acetylcholine at 2–3 months of age, at a time when the effects of splanchnic stimulation have become more pronounced. If it is assumed that during nerve stimulation the initial output of the transmitter is sufficiently high to excite many of the noradrenaline cells which are not directly innervated, the decline in the rate of noradrenaline secretion on continuous stimulation would be explained by a gradual decrease in the amount of transmitter liberated by each nerve impulse. However, certain anomalies remain; it is not easy to understand why transmission should fail so readily with successive stimulations at 15–20 min intervals, for the output of noradrenaline drops even under these circumstances. It is possible that in the young animal with a cut splanchnic nerve only a limited amount of transmitter is available, and that at relatively high rates of stimulation

the output rapidly falls to a level which is too low to excite the majority of non-innervated cells. The hypothesis also offers no explanation for the results with calves under one week of age. In these animals the small secretory response elicited by nerve stimulation suggests that innervation may be incomplete at this stage, yet there was no evidence for the increased sensitivity to acetylcholine which would be expected under these circumstances. There is also the problem of the low output of adrenaline compared with its relatively high concentration in the medulla. The apparent inability to release much of this amine either in response to nerve stimulation or to acetylcholine suggests that much of the adrenaline extractable from the glands may not be immediately available for release.

SUMMARY

1. The pressor amine content and secretory capacity of the adrenal gland were determined in calves aged from 3 hr to 220 days, and in pregnant and non-pregnant cows 2–3 yr old.

2. In the calves the percentage of adrenaline in the glands rose with age from 51 to 75 %, while 77 % was found in the adult. The total adrenaline per gland rose sharply from about the tenth day of life, while the noradrenaline content remained constant during the period investigated.

3. In the first fews days of life the output of pressor amines during stimulation of the peripheral end of the splanchnic nerve was only 2–3 times the resting level of secretion.

4. In all older calves a brief period of splanchnic stimulation resulted in the liberation of large amounts of both amines, which increased with age. The proportion of noradrenaline secreted was invariably higher than that in the glands and was unaffected by altering the frequency of stimulation.

5. Prolonged splanchnic nerve stimulation led to a rapid fall in the output of noradrenaline, while the decline in adrenaline output was less pronounced and slower in onset. This resulted in an increase in the percentage adrenaline in the effluent blood during the stimulation period. Essentially the same results were obtained when the nerve was excited intermittently.

6. The percentage of adrenaline secreted in response to splanchnic nerve stimulation in the adult was only about 5 % below that in the glands. The output of both amines fell with prolonged stimulation, but the proportions remained unchanged.

7. In the calf a higher proportion of noradrenaline was released by the intra-arterial injection of acetylcholine than by nerve stimulation. The response to acetylcholine at the end of 40 min splanchnic stimulation

was as high as that at the beginning of the experiment, which suggested that transmission failure rather than lack of available amines was primarily responsible for the fall in output during prolonged stimulation.

8. The anomalies in the development of adrenal medullary function in the calf are discussed, and a hypothesis is advanced which could explain many of the discrepancies between output of adrenaline and noradrenaline during nerve stimulation and acetylcholine injection, and the glandular concentration of these amines.

I should like to thank Dr R. S. Comline for advice and criticism, Dr D. A. Titchen for help with the cows, and G. Baker, M. Smith and P. Hughes for technical assistance. This work was begun during the tenure of a personal research grant from the Agricultural Research Council, by whom part of the expenses were defrayed.

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THE MECHANICAL PROPERTIES OF RELAXING MUSCLE

BY B. R. JEWELL AND D. R. WILKIE
From the Department of Physiology, University College London

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The response of a muscle to stimulation is classically divided into two phases: contraction and relaxation. The mechanical properties of the muscle in the first phase have been extensively studied (for references, see Jewell & Wilkie, 1958) and much is known about the way in which tension is developed and work is done, but the second phase is much less clearly understood. A. V. Hill (1949*b*) defined relaxation simply in terms of the external mechanical changes in the muscle, as 'the process by which the muscle returns, after contraction, to its initial length or tension'.

Originally our interest in relaxation was aroused by the marked difference between the time courses of isotonic and of isometric twitches. For instance, in Fig. 1, one is confronted by the apparently paradoxical situation that after having lifted a load of 3 g wt. the muscle has completely relaxed at a time when it could have borne tension of 5 g wt. under isometric conditions. Admittedly, with small loads like this, the situation is complicated by an additional factor, for in the isotonic case the muscle has shortened appreciably. However, even with large loads, where the isotonic shortening is much less, mechanical activity has apparently ended at a time when the isometrically contracting muscle is still capable of bearing a considerable tension.

METHODS

All the experiments were performed on frogs' sartorii (English *Rana temporaria*), kept immersed in Ringer's solution (mM: NaCl 115·5; KCl 2·0; $CaCl_2$ 1·8; Na phosphate buffer, pH 7·0, 2·0) at 0° C. Isotonic or isometric recordings alone give an incomplete picture of the changes in the mechanical state of the muscle, so we decided to record length and tension changes simultaneously during various different types of contraction. The length changes were recorded in the usual way by connecting the muscle to a light lever, the movements of which were 'sensed' by a photo-electric arrangement (see Jewell & Wilkie, 1958 for further experimental details). In theory the tension in the muscle could have been measured simultaneously and directly in one of three ways: by attaching a suitable transducer to the pelvic muscle clamp; by incorporating a transducer in the connexion to the muscle; or by mounting a transducer at the tip of the lever. The mechano-electronic transducer, RCA 5734, which we normally use to measure tension, cannot conveniently be immersed in Ringer's solution; and to have mounted it at the tip of the lever would have produced an undesirably

large increase in inertia. Instead of building entirely new apparatus (see, e.g., Buchthal, Weis-Fogh & Rosenfalck, 1957; Abbott & Mommaerts, 1958) we have employed an indirect method which entailed only a slight alteration to our normal apparatus (see Fig. 2).

The transducer is attached by a flexible but inextensible link to the mid point of the isotonic lever. As the transducer is fixed to the stand holding the lever, the link enables it to function as an after-load stop. When the lever is not moving

$$F = 10T + 20P,$$

where F = weight of the load, T = tension recorded by transducer and P = force produced by muscle. As F is a known constant, a record of the variation of T with time will, if suitably calibrated, show the variation of P with time.

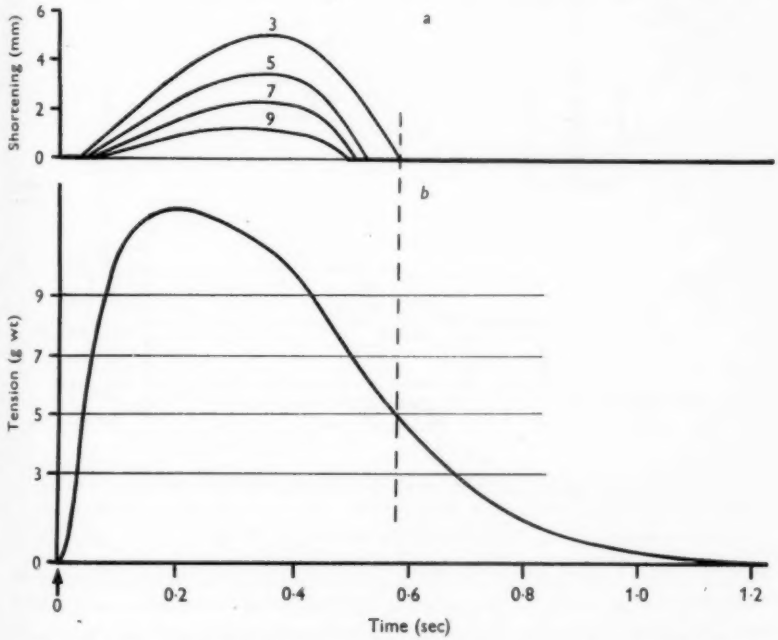

Fig. 1. Isometric and isotonic responses of a frog sartorius (30 mm, 35 mg, 0° C) to a single shock. These are tracings of experimental records, showing (*a*) after-loaded isotonic contractions against loads of 3, 5, 7 and 9 g wt., and (*b*) an isometric twitch; the horizontal lines correspond to the loads in (*a*).

The lever can only move in such a direction that the link between it and the transducer goes slack; this will happen whenever the force produced by the muscle is greater than that produced by the load. When this happens, T will become zero, and then

$$P = F/20 + Ma,$$

where M is the equivalent mass of the lever at some chosen point, and a is the acceleration at the same point. With the lever actually used, Ma was always a negligible quantity; so that the force on the shortening or lengthening muscle was always equal to one twentieth of the load. Thus the force exerted by the muscle was known at all times, whether it was shortening

or not. The outputs of the transducer and photocell (which registers movements of the lever) were amplified and displayed simultaneously on a single-beam cathode-ray tube, by means of mechanical input switching at 250 c/s.

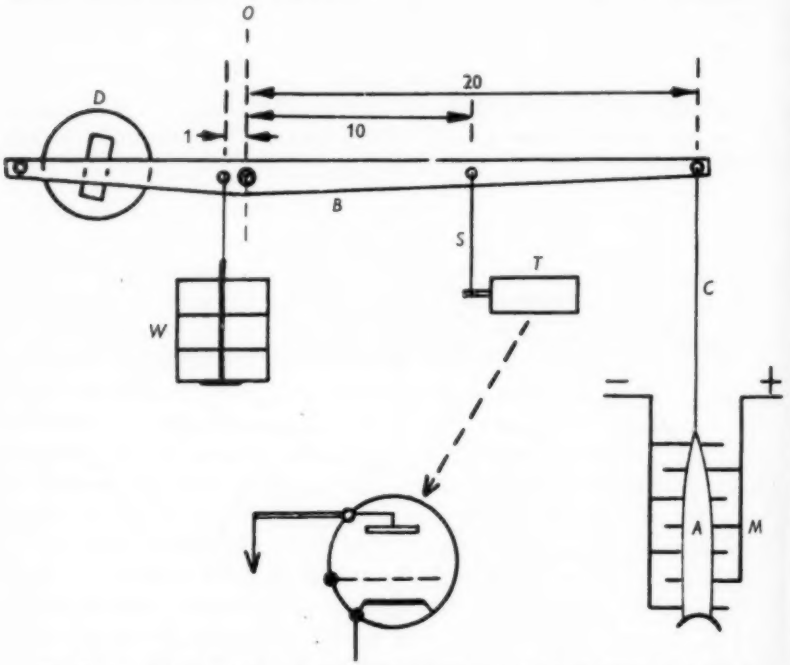

Fig. 2. Apparatus used for making simultaneous records of the length and tension changes in the muscle. The frog sartorius (*A*) lies on a multi-electrode assembly (*M*) in oxygenated Ringer's solution. It is attached to the light isotonic lever (*B*) by means of a stainless-steel connexion (*C*). The lever is pivoted at (*O*), and its movements are recorded by means of the photo-electric device (*D*). Weights (*W*) are hung on the lever near its fulcrum, and the length that the muscle adopts is determined by the position of the transducer (*T*), which is attached to the lever by means of a flexible, but inelastic, connexion (*S*). The numbers give the relative distances of the load, the transducer, and the muscle from the fulcrum of the lever.

RESULTS

Figure 3 shows a typical series of records of the length and tension changes in the muscle during after-loaded isotonic contractions against various loads. It is clear from the records that the rapid extension of the muscle as the load drops does not mark the end of the process of relaxation, for this is followed by a phase of isometric relaxation during which the tension first drops rapidly, and then continues to decay at a rate which is similar to that in an isometric twitch (see Fig. 1*b*). The exponential

character of the slow fall is seen clearly if the logarithm of the tension is plotted against time, as in Fig. 6*a*. The fact that these semi-logarithmic plots all have the same slope means that the tension decay after isometric contraction and after isotonic contractions against various loads can be described by the same decay constant (half-time or time constant). Thus, although the absolute tension at a given moment after the stimulus depends on the mechanical changes that the muscle has undergone, the rate of proportional fall of tension is independent of these changes. The relation between tension and mechanical change can be expressed quantitatively

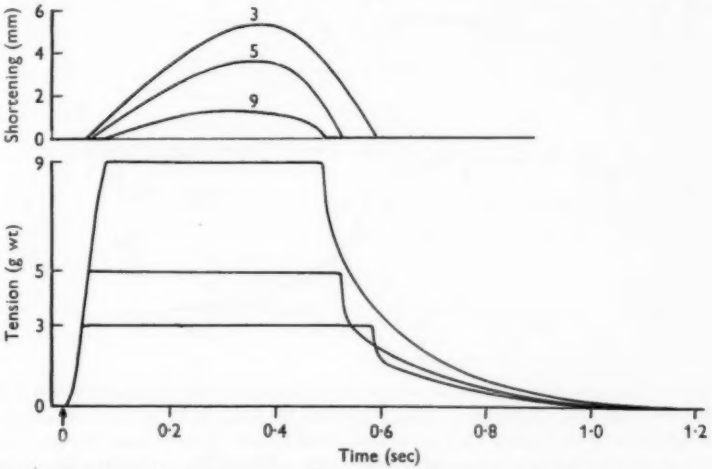

Fig. 3. Tracings of simultaneous records of the length and tension changes in a frog sartorius (30 mm, 5 mg, 0° C) during after-loaded isotonic twitches against loads of 3, 5, and 9 g wt. The corresponding isometric twitch is shown in Fig. 1*b*.

in a variety of ways, depending on whether one is mainly interested in the history of changes in length or in tension. During ordinary isotonic contraction the relation is particularly simple, as each millimetre of active shortening reduces the tension at a given later moment to about 3/4. Alternatively, as the fall of tension is exponential, each millimetre of shortening can be considered to shift the curve of fall of tension forwards by about 70 msec.

In contrast, the initial sudden drop in tension seems to be correlated with, and may be a consequence of, the sudden *increase* in length imposed by the falling load; for the size of this step is greater the greater the lengthening of the muscle. This effect may be discerned in Fig. 3; but as both the load and the forced lengthening of the muscle were being changed in this experiment, it is not clear which factor was responsible for the progressive increase in the size of the step.

To overcome this difficulty we have performed a separate experiment in which the load was kept constant at 6 g wt., but the amount of lengthening during relaxation was varied (see Fig. 4). This was done by mounting the transducer on the armature of an electromagnet so that it could be moved to a new position while the contraction was actually proceeding. The difference between the initial and final lengths of the muscle could be varied by adjusting the excursion of the armature of the electromagnet. Figure 4 shows clearly that as the amount of forced lengthening during

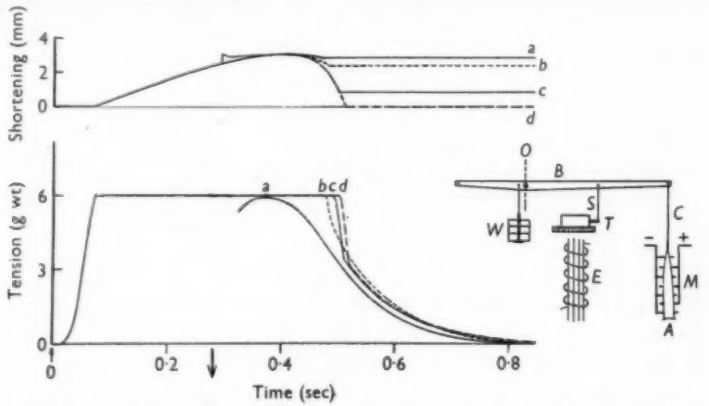

Fig. 4. Tracings of simultaneous records of the length and tension changes in a frog sartorius (30 mm, 35 mg, 0° C) during after-loaded isotonic twitches, in which the lengthening of the muscle during relaxation was varied. In each case, the load was 6 g wt. and the shortening during contraction was 3·1 mm. The subsequent amounts of lengthening were: (*a*) 0·28 mm, (*b*) 0·81 mm, (*c*) 1·36 mm, and (*d*) 3·1 mm. The apparatus used is shown as an inset diagram; the lettering is the same as in Fig. 2. The transducer (*T*) was mounted on the armature of an electromagnet (*E*) so that it could be moved downwards whilst the muscle was shortening. The timing of the movement is shown by the arrow below the time scale.

relaxation is decreased, the size of the sudden drop in tension also decreases. It is just as though, in an ordinary isotonic contraction, the rapid extension imposed by the falling load caused a further fall in tension by breaking some of the tension-bearing bonds. The effect of a rapid extension was later examined in a more direct fashion by applying a small and rapid stretch to the muscle during the later stages of an isometric twitch (Fig. 5*d*). This manœuvre leads to an increase in tension during the period of the stretch, but this is followed immediately by a sudden fall in tension, and then by an exponential decay; that is, the consequences are exactly the same as those of forced lengthening during an isotonic contraction.

Similar behaviour was shown by the relaxing muscle in all the types of

contraction that we have examined. These results are grouped together in Fig. 5, which shows simultaneous records of length and tension during isotonic contractions; isometric contractions, with and without stretches or releases; stopped isotonic contractions; and auxotonic contractions, in which the muscle shortened against a spring. Semi-logarithmic plots of the tension decay, corresponding to the curves in Fig. 5, are shown in Fig. 6. In all cases the tension record shows a final exponential fall. The only exception (not shown in Fig. 5), is seen when the relaxing muscle is quickly

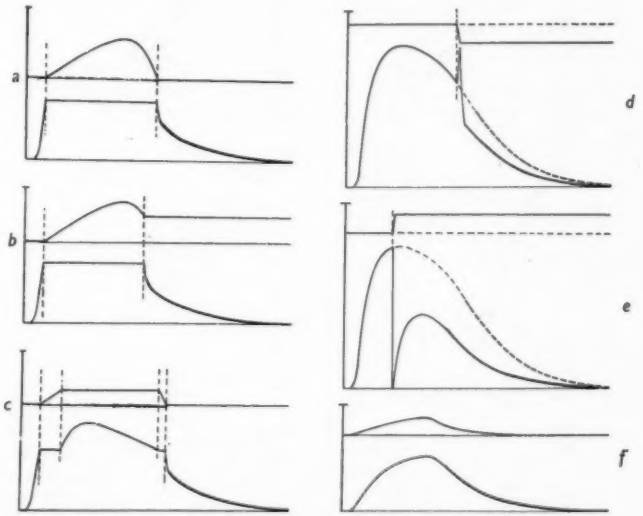

Fig. 5. Simultaneous records of length change (upper line) and tension change (lower line) in various types of muscle twitch. In each case, the final exponential tension fall has been darkened, for emphasis. *a*, after-loaded isotonic; *b*, after-loaded isotonic, load held up; *c*, after-loaded isotonic, arrested by a stop; *d*, isometric, stretched during relaxation; *e*, isometric, released during contraction; *f*, auxotonic, contracting against a spring.

stretched and then restored again to its original length. After this treatment, tension remains zero, presumably because the muscle cannot shorten enough to take up the slack resulting from the stretch.

In each of the graphs shown in Fig. 6 the tension decay in a simple isometric twitch (recorded from the same muscle at about the same time) has been included to provide a standard of reference (solid dots); and in each case this is seen to have about the same slope as the other curves of tension fall. The slopes are not the same in the different parts of Fig. 6 because the experiments shown there were made on different muscles, or on the same muscle at different times. However, the inclusion of a control

isometric twitch in each set permits the deduction that, if all the experiments could be made on the same muscle at the same time, they would *all* show the same slope. Thus the time constant of tension decay appears to be characteristic of a particular muscle under given conditions.

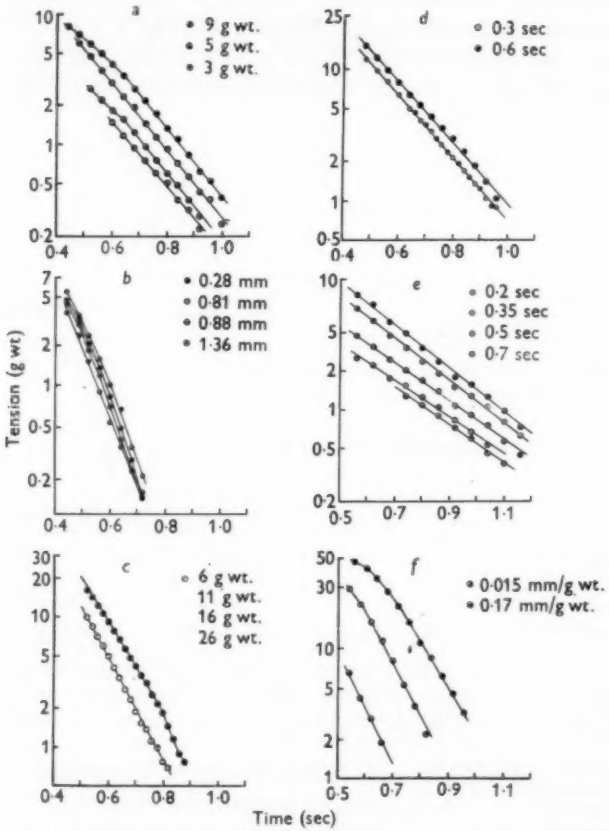

Fig. 6. Semi-logarithmic plots of the fall of tension during relaxation in the various types of contraction shown in Fig. 5. In each case the solid circles show the fall of tension in an isometric twitch. The open circles show the fall in (*a*) after-loaded isotonic contractions against loads of 3, 5 and 9 g wt. (see Fig. 3); (*b*) after-loaded isotonic contractions against a load of 6 g wt., in which the change in length during relaxation was varied from 0·28 to 1·36 mm (see Fig. 4); (*c*) after-loaded isotonic contractions, in which the shortening was limited to 1 mm (stopped contractions)—the tension fall was identical for all loads used (6, 11, 16, and 26 g wt.); (*d*) isometric contractions, in which the muscle was stretched by 1 mm at 0·3 and 0·6 sec after the shock; (*e*) isometric contractions, in which the muscle was released by 1 mm at various times after the shock; (*f*) auxotonic contractions, in which the muscle shortened against springs with different compliances.

By changing these conditions the decay constant can be altered. For instance, increasing the temperature decreases it, the Q_{10} being about 3 (also M. C. Goodall, 1958, unpublished), and raising the hydrostatic pressure increases it (Brown, 1936). Alteration in the length of the muscle is known to produce a marked effect on the decay constant (see Hartree & Hill, 1921). This is illustrated in Fig. 7, in which the effect of muscle

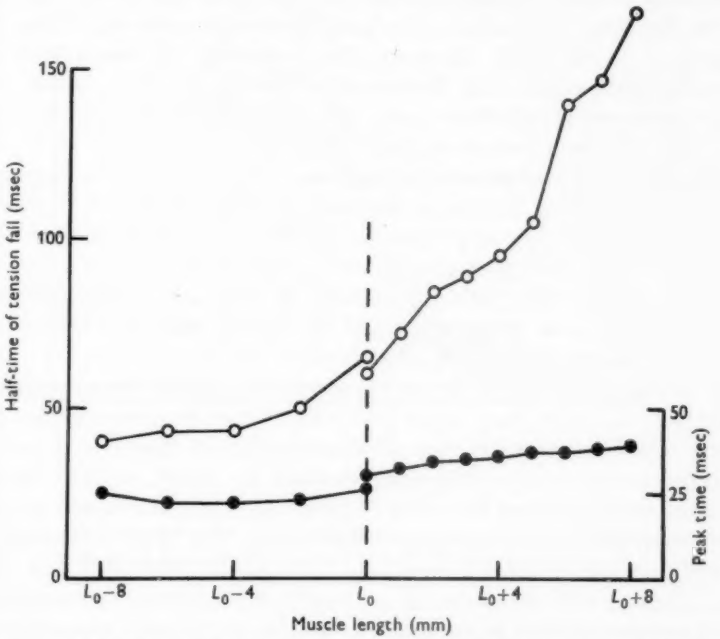

Fig. 7. Graphs showing the effect of varying the muscle length on the time course of the isometric twitch. The solid circles show the peak time, and the open circles show the half-time of the tension fall, in isometric twitches of a frog sartorius (60 mg, L_0 = 29 mm, 0° C) at muscle lengths from L_0-8 to L_0+8 mm.

length on the peak time of the isometric twitch is also given for comparison. At lengths below L_0, there is little effect, but when the muscle is stretched the decay constant increases steeply, while the peak time is only slightly affected. Relaxation always becomes slower during the course of a long experiment; for instance, in a typical case, the half-times on three successive days were 66, 113 and 180 msec.

The mechanical state of the relaxing muscle

Muscle undoubtedly contains a series compliance, with properties that remain more or less unchanged up to the end of relaxation (Wilkie, 1956;

Jewell & Wilkie, 1958). This compliance must shorten and stretch out the contractile elements as the tension falls during relaxation. The first possibility to be considered is whether the mechanical properties of relaxing muscle can be explained by means of a purely passive Maxwellian system of springs and dashpots. An exponential decay of tension would be given by a linear stretched spring and a dashpot connected in series; so it is tempting to draw analogies between the spring and the series compliance on the one hand and between the dashpot and the (relaxing) contractile component on the other. However, this possibility has been tested, and rejected, by the experiment illustrated in Figs. 5*f* and 6*f*, in which extra compliances were introduced into the connexion between the muscle and the transducer. In the hypothetical Maxwellian system, this would have led to an increase in the time constant of the tension decay, whereas in the muscle there was little effect. The simple Maxwellian system is therefore ruled out, and so, probably, are other passive mechanical systems of that type. The viscosity of the relaxing contractile component evidently varies in a complicated way with both the velocity of lengthening and with time; and although these properties could be incorporated in a Maxwellian model, the result would be of purely formal use, if any.

However, the presence of two elements does complicate experimental investigations of the time course with which active processes decline after stimulation. The simplest way of separating their effects is to use the quick-release technique originally devised by Petit (1931). After a stimulus, the muscle first contracts isometrically; then, at a chosen moment, it is released and allowed to shorten isotonically. The length changes in the muscle show two distinct phases (see, for example, Jewell & Wilkie, 1958, Fig. 3); the initial rapid change in length arises from shortening of the series elastic elements as the tension on them is reduced; this is followed by a much slower change in length as the contractile parts of the muscle continue to shorten actively. It is found experimentally that the sharp distinction between the two phases remains clear throughout contraction and relaxation (see Fig. 8). One can therefore examine the contractile component alone by subtracting the contribution of the series compliance.

It is found that the active shortening derived in this way continues almost to the end of relaxation, though the amount of shortening, its speed, and the force that can be overcome, all decrease progressively. Although these changes could be put into quantitative form in a variety of ways, it must be remembered that all the individual muscle fibres may not behave in an identical fashion (see Discussion). We have determined the force–velocity curve of the contractile component at each moment of release by measuring the velocity with which the muscle shortens actively against different loads. Figure 9 shows the force–velocity curves that were

obtained at various times after the application of a single shock to the muscle. All these curves could probably have been fitted by Hill's equation (Hill, 1938), but we have found it convenient to use one of the forms of Aubert's equation (Aubert, 1956*a*), which lends itself to the ready determination of P_0 (the force when the velocity is zero) and V_0 (the velocity of shortening when the load is zero), as shown in Fig. 10. If less

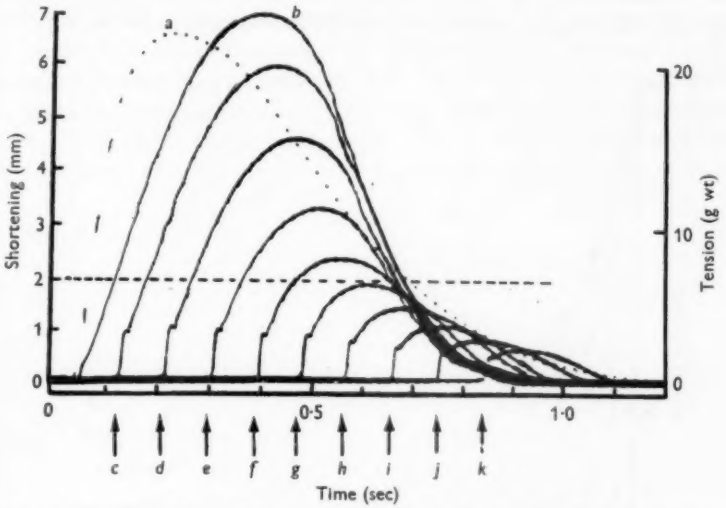

Fig. 8. Superimposed experimental records showing the response of a frog sartorius (34 mm, 63 mg, 0° C) to a single shock (*a*) under isometric conditions, and (*b*) under after-loaded isotonic conditions. Records *c*–*k* show the changes in length that occurred when the muscle was released and allowed to shorten under isotonic conditions at various times during an isometric twitch; in these records the initial rapid elastic changes in length were too faint to reproduce satisfactorily, and they have therefore been retouched. All the isotonic contractions (*b*–*k*) were made against a very small load (i.e. not exceeding 0·3 g wt.). The horizontal interrupted line has been added to show a length change of 2 mm (see text).

accuracy is needed, P_0 and V_0 can be directly estimated from sets of releases (like that shown in Fig. 8) against a range of loads. The way in which V_0 and P_0 decline after a single shock is shown in Fig. 11, with an ordinary isometric twitch for comparison. It appears that V_0 declines rather more slowly than P_0.

A variety of other measures of the decline in activity can be devised, such as the maximum power output, the maximum work output, or the maximum total shortening against a very small load. Since maximum power output is approximately equal to $P_0 V_0/9$ (Hill, 1939*b*), this declines in a way that is intermediate between the curves for P_0 and V_0. With work

output, or total shortening, there is the difficulty that each takes place over a finite interval of time rather than at a single instant, so it is not quite clear how the result should be plotted for a fair comparison with the other curves. The only point in mentioning them is to emphasize that all these curves are different, and no particular one can claim to be *the* index of declining activity. The same is true in the case of the 'active-state curve' obtained by Ritchie's method (Ritchie, 1954), in which the isometrically contracting muscle is released a fixed distance (usually 2–3 mm)

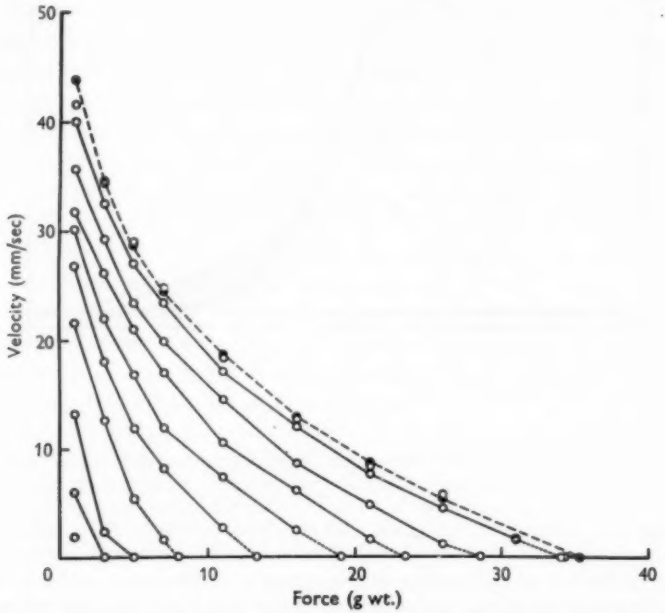

Fig. 9. The relationship between the load and the velocity of shortening of a frog sartorius (32 mm, 56 mg, 0° C) at various times after a single shock. The solid circles show the shortening velocities in after-loaded isotonic contractions against various loads; these have been joined by an interrupted line. The open circles were obtained by measuring the velocity of active shortening when the muscle was released against different loads at predetermined times after the shock (Fig. 8 shows a typical set of releases against one particular load). A force–velocity curve was obtained for each of these times by drawing a line through the corresponding set of points. For the sake of clarity, a line has not been drawn through the points obtained from releases made 92 msec after the shock, as these are almost identical with those obtained in after-loaded contractions. The other curves correspond to releases made 184, 276, 368, 460, 552, 644, 736, 828 and 920 msec after the shock. Some of the curves were incomplete in that they lacked an isometric point; these curves were fitted by Aubert's equation (as described in Fig. 10), and the isometric points were obtained by extrapolation. The parts of the curves obtained in this way are shown by dotted lines.

at various times, and its capacity to redevelop tension is recorded. A curve obtained by this technique (using a 2 mm release) is included in Fig. 11 (curve *B*) for comparison with those for P_0 and V_0. The main point to notice is that the 'active-state' curve and the 'P_0' curve run together at first, when the tension is high, but that the active-state curve soon separates and drops away towards the base line. This is puzzling, because both curves are supposed to measure the isometric tension that the muscle can develop at various moments after the stimulus. Indeed, the 'P_0' curve must, logically, be identical with the 'active-state' curve that would be obtained if the amount of release in the latter case could be adjusted at each moment of release so that it was only just enough to discharge the tension in the series elastic component. This is impracticable and in the past it has been the custom to allow a generous margin to accommodate

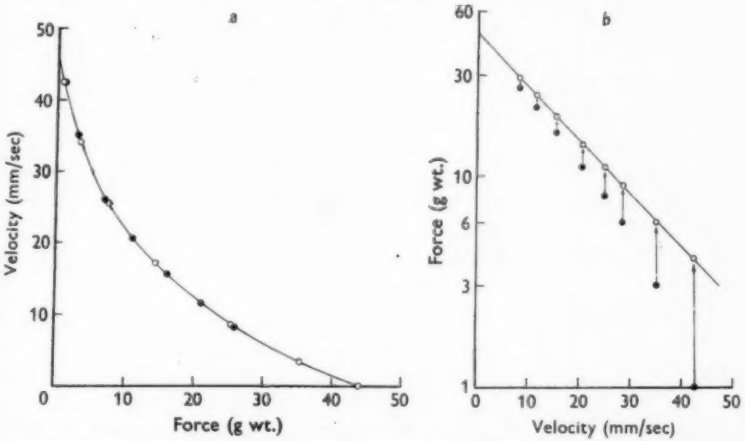

Fig. 10. Graphs illustrating the use of Aubert's equation for fitting the force–velocity curve, and for predicting a value of the isometric tension in the absence of suitable experimental points at low shortening velocities. The equation used (Aubert, 1956*a*) is $P = A \exp(-V/B) \pm F$, where P = force, V = velocity, and A, B and F are constants. The experimental points (solid circles) have been plotted on both linear and semi-logarithmic co-ordinates (graphs *a* and *b*, respectively). A constant force, F (= 3 g wt.), has been added to these points to give a straight line on the semi-logarithmic plot (open circles in graph *b*). The constants A and B can then be evaluated by extrapolating the straight line to zero velocity (which gives A = 47 g wt., from the intercept on the ordinate), and by measuring its slope (which gives B = 17 mm/sec). Since F = 3 g wt., the equation of the force–velocity curve (i.e. solid circles) is $P = 47 \exp(V/17) - 3$, where P = force in g wt., and V = velocity in mm/sec. This equation has been solved for various values of V and the points obtained have been plotted on graph *a* as open circles; it is evident that the fit is extremely good. Values for P_0, the isometric tension, and V_0, the maximum velocity of unloaded shortening, can be obtained by solving the equation for $V = 0$ and $P = 0$, respectively. In this particular case, P_0 = 44 g wt. and V_0 = 44·5 mm/sec.

uncertainties in the series compliance of the muscle and apparatus, and to make sure that both were discharged fully even when the tension was at its peak. The consequence of this policy is shown in Fig. 8, in which a horizontal line has been drawn to indicate a shortening of 2 mm. In Ritchie's method, using a 2 mm release, tension will only begin to be recorded *after* the shortening curve has crossed this line, and it is evident that this will never happen if the release is made after 0·4 sec, for the maximum unloaded shortening of the muscle is then insufficient to take up the slack in

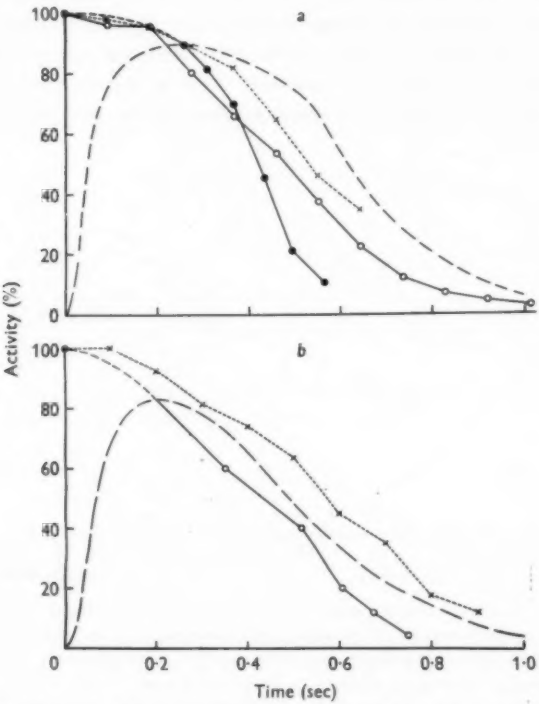

Fig. 11. Time course of the decay of activity in the frog sartorius after a single shock. In both graphs, 100% indicates full activity; it corresponds to P_0, the isometric tetanic tension, in the case of the isometric measurements; and to V_0, the maximum velocity of shortening of the unloaded muscle, in the case of the isotonic measurements. The interrupted curves show isometric twitches and the solid circles in (*a*) show the active-state curve that was obtained by Ritchie's method using a 2 mm release. The other curves show the decline of activity as measured by the isotonic release method: open circles, decline of P_0; crosses, decline of V_0. (*a*) Frog sartorius (32 mm, 56 mg, 0° C); P_0 and V_0 determined by fitting the force–velocity curves at various times after a single shock (Fig. 9) by Aubert's equation (Fig. 10); (*b*) frog sartorius (34 mm, 63 mg, 0° C); P_0 and V_0 determined by direct measurement from experimental records (see Fig. 8).

the connexion. For this reason, Ritchie's method will tend to underestimate the duration of activity in the muscle. It should be possible to reduce this error by decreasing the amount of release; and this is found to be the case experimentally (Fig. 12). With releases of from 2 to 5 mm, the shape of the curve is unaltered in its upper part (see also Ritchie & Wilkie, 1955, p. 495); but when the release is reduced to 0·5 mm the bottom end of the active-state curve is drawn to the right so that it coincides with the 'P_0' curve (compare Figs. 11 and 12). A similar effect has been found by M. C. Goodall (1958, private communication). In fact it is only the bottom end of the curve that is appreciably affected in this way, and since the

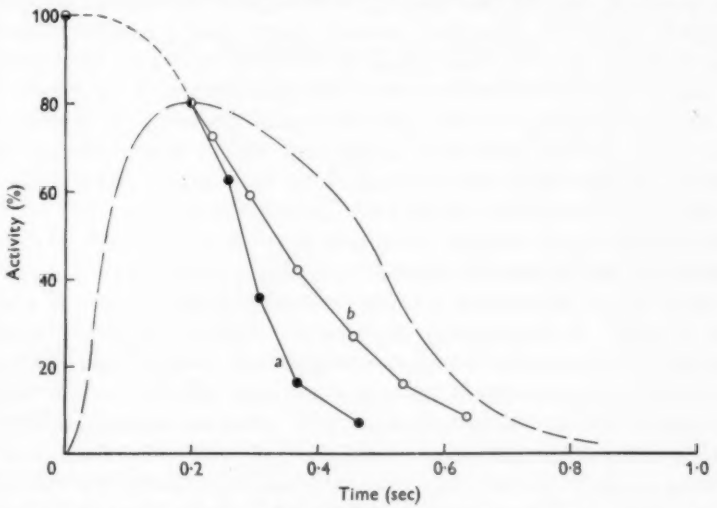

Fig. 12. Active-state curves of a frog sartorius (31 mm, 39 mg, 0° C) determined by Ritchie's method, using releases of (*a*) 2 mm, and (*b*) 0·5 mm. On the ordinate scale, 100 % corresponds to the isometric tetanic tension. The dashed curve shows the time course of the tension changes in an isometric twitch.

active-state curves already published (Ritchie, 1954; Lammers & Ritchie, 1955; Ritchie & Wilkie, 1955) do not extend to late times, they are probably reasonably correct as they stand, for in each of these cases the point of interest was whether the curve as a whole was advanced or delayed. For this purpose, Ritchie's technique (even using a large release) is adequate, and convenient.

DISCUSSION

Properties of relaxing muscle

In describing the results of our experiments we have made use of the well known fact that the curve of fall in tension during isometric

relaxation eventually becomes exponential. Although there is some variation between muscles, this exponential phase usually includes from 50 to 70% of the total fall in tension. In order to fit the complete curve of fall in tension in isometric tetanus, a different mathematical function is needed—for example, a probability curve (Aubert, 1956*b*), or a more complicated function involving several parameters (M. C. Goodall, private communication).

The ability to bear tension usually outlasts the ability to shorten actively, even against zero load, and the ability to generate mechanical power declines even sooner. All these signs of activity disappear very rapidly once active shortening has been permitted, and the muscle behaves as though it contains a dwindling store of energy that is rapidly exhausted by shortening. In some other types of muscle there is a far more striking dissociation between the ability to shorten actively and the ability to bear tension; for instance, in the anterior byssal retractor of *Mytilus*, the capacity for active shortening disappears within a few seconds after stimulation has ended, but the ability to bear tension may persist for several hours (Lowy & Millman, 1959; Jewell, 1959).

No matter what changes in length may have occurred, the decay constant of fall in tension remains surprisingly constant, in view of the fact that active shortening considerably reduces the tension at a given later moment. It is tempting, therefore, to identify the decay constant with the rate constant of one of the fundamental chemical reactions during relaxation; though at the moment it is not clear exactly how our findings on living muscle are to be compared with those on relaxing actomyosin systems. One of the advantages claimed by Goodall's formulation of the problem is that it can be interpreted in terms of the kinetics of the underlying reactions. Unfortunately, we cannot see how to test this formulation against our experimental results, though the results themselves agree with his, where they can be directly compared.

If the tension during relaxation is really held by bridges between actin and myosin filaments (H. E. Huxley, 1957; A. F. Huxley, 1957), then during forcible lengthening all the bridges must be broken as the filaments slide past one another, but must re-form immediately when elongation ends. This may not be as remarkable as it sounds, because the contact area between the interdigitating filaments is enormous (about 200,000 cm^2 per cm^3 of muscle, i.e. 30 cm^2 per cm^2 in the half-sarcomere). The force between the filaments is consequently small (less than 100 g wt. per cm^2 of contact area), and it may be compared with ordinary limiting friction.

One practical conclusion that we have drawn from our experiments is that in order to obtain a true picture of the mechanical state of the muscle, it is always necessary to record, to define, or at least to consider the simul-

taneous changes in length and tension. Many workers (e.g. Blix, 1895; Reichel, 1936; see also Burdon-Sanderson, 1900) have done this in the past, though their records were often unsatisfactory because of the inadequate frequency-response of their purely mechanical apparatus and the fact that their experiments were usually conducted at room temperatures. More recently, electro-mechanical apparatus has been used to overcome this difficulty (see Buchthal *et al.* 1957; Abbott & Mommaerts, 1958) and experiments have usually been made at lower temperatures. It is of vital importance in the interpretation of heat measurements to take both the tension and length changes in the muscle into consideration, even if they cannot actually be recorded simultaneously.

Heat production during relaxation

Although heat is produced throughout contraction and relaxation, that due to active processes (processes that consume chemical energy) is thought to end about 0·4 sec after the application of a single shock to the frog sartorius at 0° C. The heat production during this period is believed to be of two distinct types (Hill, 1949*a*)—(i) 'activation' heat, which inevitably accompanies the creation of an active state in the muscle, and (ii) 'shortening' heat, which is proportional to the amount of shortening in the contractile component. The latter is regarded as the result of active processes because its appearance is accompanied by additional recovery heat (Hill, 1939*a*) and, probably, by additional oxygen consumption (Fischer, 1931). The heat produced after 0·4 sec can be accounted for by purely passive processes, viz. (i) the degradation of the mechanical energy that was stored, during contraction, either in the series elastic elements or in the load (Hill, 1949*b*; Abbott & Aubert, 1951) and (ii) the thermo-elastic heat that accompanies the tension fall, as in a normal elastic body (Hill, 1953, 1959).

Now, it has been shown in this paper that the muscle can still shorten actively after 0·4 sec. In fact, it can shorten against zero load for at least 1 sec (see Fig. 8), and the question arises whether or not this is accompanied by the production of shortening heat. If it is, then the view that active processes cease at 0·4 sec needs revision; on the other hand, if it is not accompanied by shortening heat, then one must presume that the nature of the shortening produced by the muscle changes qualitatively during the course of a twitch, in spite of the fact that the mechanical records (Fig. 8) and the force–velocity curves (Fig. 9) give no indication of this. A difficulty that arises in the interpretation of mechanical records of this kind is that there is no way of knowing whether all the muscle fibres relax simultaneously. If they do, then all of them must be active at late times. If they do not, then the relaxation of the whole muscle must be to

some degree determined by the statistical distribution of relaxation times in the different fibres; moreover, a few active fibres would suffice to produce shortening against zero load at late times. In this case, even if the shortening heat per fibre had its usual value, the shortening heat produced by the whole muscle might be negligible.

SUMMARY

1. A simple indirect method has been used to record simultaneous changes in length and tension in a frog's sartorius muscle during various types of muscle twitch.

2. In all cases the fall in tension eventually becomes exponential and the decay constant was found to be independent of the sequence of changes in length and tension that the muscle had undergone.

3. An isotonic quick-release technique has been used to follow the decline in activity in the muscle.

4. The use of various measurements for assessing activity is discussed. According to some criteria, activity persists at 0° C for as long as 0·9 sec after a shock.

5. The question of the heat production at late times is discussed.

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TRANSFER OF SHORT-CHAIN FATTY ACIDS BY THE INTESTINE

By R. J. C. BARRY and D. H. SMYTH
From the Department of Physiology, University of Sheffield

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Smyth & Taylor (1958) have shown that acetic, propionic and butyric acids can be transferred against a concentration gradient by preparations of rat intestine *in vitro*. These studies were limited to observations on the concentration of fatty acid in mucosal and serosal fluids and to measurement of changes in the amount of fatty acid and water on the serosal side. The present work extends these observations to further details of movement of these fatty acids and to higher members of the fatty acid series. The results also provide a basis for discussing the interpretation of intestinal transfer studies *in vitro*.

METHODS

In vitro. The preparation was the sac of everted small intestine of the rat (Wilson & Wiseman, 1954). The sacs were 30 cm long, made from the jejunum of the rat, contained 3 ml. bicarbonate saline (Krebs & Henseleit, 1932) with 500–1000 mg glucose/100 ml., and were suspended in 15–50 ml. of the same solution. They were shaken at a rate of 80 c/min for 60 min at 38° C in conical flasks, as described by Parsons, Smyth & Taylor (1958). Two types of experiments were done as also described by these authors: (1) transfer experiments, in which the object of the experiment was to measure the changes in the mucosal and serosal fluids; and (2) metabolism experiments, where the object was to estimate the total amount of fatty acid disappearing during the course of the experiment. In the metabolism experiments, the method was modified as follows. After incubation of the sac for 1 hr the flask was immersed in an ice-salt freezing mixture (approximately $-10°$ C) until the contents froze (approximately 3 min). Cold water was then run over the outside of the flask until the contents just thawed. The sac was removed and ground with 1 ml. of acid-washed sand, 5 ml. 7 % (w/v) $CuSO_4 . 5H_2O$ and 5 ml. 10 % (w/v) $Na_2WO_4 . 2H_2O$. The ground-up tissue together with the deproteinizing reagents was pooled with the flask contents, and the total volume made up to 100 ml. After filtering, samples were taken for estimation of volatile fatty acid.

Expression of results. The terms used in expressing the results are as defined by Parsons *et al.* (1958), and for convenience they are repeated here. 'Mucosal fluid transfer' is the decrease in volume of the mucosal fluid; 'serosal fluid transfer' is the increase in volume of the serosal fluid; 'gut fluid uptake' is the increase in volume of the empty sac; 'mucosal substrate transfer' is the decrease in amount of substrate (in this case fatty acid) on the mucosal side; 'serosal substrate transfer' is the increase in amount of substrate on the serosal side. 'Final concentration gradient' is the difference between the final serosal substrate concentration and final mucosal substrate concentration. In addition to the above

terms the following are also used: 'gut substrate uptake' is the amount of substrate not accounted for in the final mucosal and serosal fluids and may be due either to metabolism or to accumulation of substrate in the intestine. 'Mucosal concentration transferred' is the mucosal substrate transfer divided by the mucosal fluid transfer, and the 'serosal concentration transferred' is the serosal substrate transfer divided by the serosal fluid transfer. If the figure of 3·2 for the wet wt.:dry wt. ratio found by Fisher & Parsons (1953*a*) is accepted, the 'initial gut fluid content' can be calculated from the initial wet weight, and if to this is added the 'gut fluid uptake', the 'final gut fluid content' is obtained. If the amount of substrate in the gut at the end of the experiment is known the 'final gut substrate concentration' can be calculated. The above quantities as defined are considered as positive. Under certain conditions some of them may become negative. In comparing the results of different experiments it is usually convenient to express the transfer per gram initial wet weight.

When many experiments are done, the calculation of all the above quantities becomes laborious. The work has been greatly facilitated by the use of the Pegasus Electronic Computer. A programme for the work was prepared by Mr P. H. Blundell, and this very greatly reduced the time spent on calculation.

In vivo. The method for studying absorption *in vivo* was that of Sheff & Smyth (1955), as modified by Jervis, Johnson, Sheff & Smyth (1956). Incubation of a sample of mucosal fluid containing glucose did not show any production of volatile fatty acid, thus excluding possible formation of volatile fatty acid by bacterial action.

Chemical. The fatty acids were obtained commercially and were used as sodium salts. They were estimated by steam distillation in a Markham still after deproteinization with 7% (w/v) $CuSO_4.5H_2O$ and 10% (w/v) $Na_2WO_4.2H_2O$. The final titration was with 0·05N-NaOH, with phenol red as indicator.

Statistical. In the section on Michaelis–Menten kinetics the statistical procedures used were those described by Davies & Jowett (1959).

RESULTS

Metabolism of fatty acids

The purpose of these experiments was to determine the capacity of the intestine to metabolize or produce fatty acid. The production of volatile fatty acid in the absence of added fatty acid is given in Table 1, which includes experiments in the presence and absence of glucose, and experiments where the sac was suspended in saline containing glucose but removed without having been incubated. Two experiments were also done in which lengths of intestine were extracted immediately after removal without making into a sac. These results show that incubation of the sac produces a small amount of volatile fatty acid which is possibly slightly greater in the presence of glucose. Without incubation the amount of volatile fatty acid produced is insignificant. Table 2 shows the relation between the amount of fatty acid added and the amount recovered in the presence and absence of glucose for acetate, propionate and butyrate, respectively. In the case of acetate and butyrate, fatty acid disappeared during incubation in the absence of glucose, and this disappearance is more striking if the amount of fatty acid produced in the absence of added

TABLE 1. Production of volatile fatty acid by 30 cm sacs of everted small intestine when no fatty acid was present initially. The sacs were suspended in Krebs's bicarbonate saline, with or without added glucose. In the last two experiments shown, 30 cm of intestine was ground up immediately after removal from the animal without eversion

Glucose concn. (mg/100 ml.)	Time of incubation (min.)	Volatile fatty acid (μmole)	Mean (μmole)
500	60	7·7	7·7
500	60	9·5	
500	60	6·8	
500	60	6·8	
0	60	4·7	5·4
0	60	6·0	
500	0	5·7	2·8
500	0	3·1	
500	0	1·4	
500	0	0·9	
0	0	2·4	2·6
0	0	2·8	

TABLE 2. Change in total amount of volatile fatty acid after 60 min incubation of sacs of everted small intestine in Krebs's bicarbonate saline with fatty acid added initially in the presence or absence of glucose. The initial concn. of fatty acid was approximately 10 mM in all cases. In the column marked 'difference' a positive quantity indicates production of volatile fatty acid and a negative quantity disappearance

Fatty acid	Initial glucose concn. (mg/100 ml.)	Volatile fatty acid (μmole) Initial	Final	Difference	Mean
Acetate	1000	178	178	0	− 1
	1000	178	175	− 3	
	1000	179	177	− 2	
	1000	179	180	1	
	0	179	168	−11	−13
	0	179	166	−13	
	0	180	164	−16	
	0	180	168	−12	
Propionate	1000	178	183	5	5
	1000	178	182	4	
	1000	161	167	6	
	1000	161	167	6	
	0	161	163	2	3
	0	161	162	1	
	0	178	183	5	
	0	178	182	4	
Butyrate	1000	175	172	− 3	− 5
	1000	175	172	− 3	
	1000	182	178	− 4	
	1000	182	176	− 6	
	1000	182	174	− 8	
	0	175	163	−12	−11
	0	175	167	− 8	
	0	182	169	−13	
	0	182	172	−10	
	0	182	168	−14	

fatty acid is also considered. In contrast there appeared to be almost no metabolism of propionate.

TABLE 3. Transfer of acetate and fluid by everted sac of rat jejunum. The initial wet weight of the sac was 1·98 g. Duration of experiment 60 min

Acetate (μmole)	
Mucosal transfer	161
Serosal transfer	72
Gut uptake	89
Fluid (ml.)	
Mucosal transfer	2·58
Serosal transfer	1·56
Gut uptake	1·02
Acetate concn. (mM)	
Initial mucosal and serosal	20·3
Final mucosal	11·5
Final serosal	29·2
Final concn. gradient	17·7
Mucosal concn. transferred	62·4
Serosal concn. transferred	46·2

Transfer of fatty acids

The general pattern can be seen by reference to one experiment with acetate (Table 3). The initial concentration was the same in mucosal and serosal solutions, i.e. 20·3 mM. During the experiment 161 μmole acetate and 2·58 ml. fluid left the mucosal side. The acetate concentration in the fluid leaving the mucosal side (mucosal concentration transferred) was therefore 62·4 mM i.e. about three times the initial concentration in the mucosal fluid. Of the acetate and fluid leaving the mucosal side, some was retained in the gut and some was transferred to the serosal side. The gut acetate uptake (89 μmole) was proportionately greater than the gut fluid uptake (1·02 ml.) with the result that the serosal concentration transferred (46·2 mM) was less than the mucosal concentration transferred. The serosal concentration transferred was, however, greater than the initial concentration of acetate on the serosal side, with the result that the final serosal concentration of acetate (29·2 mM) was greater than the initial serosal concentration (20·3 mM). Of the 89 μmole of acetate taken up by the tissue, presumably a small amount was metabolized and the rest retained in the gut wall. Similar results were obtained in more than fifty other experiments with acetate. Analogous results were also obtained with propionate, butyrate, *n*-valerate, *iso*-valerate and *n*-hexanoate.

Significance of serosal transfer

As a considerable amount of water and fatty acid is present in the gut wall at the end of the experiment, the relation was determined between the concentration of fatty acid in the gut wall, the final serosal concentration, the mucosal concentration transferred and the serosal concentration

transferred. Since propionate is not metabolized it was used for these experiments, Table 4 gives the results of one complete experiment for propionate, in which the amount in the gut wall was determined by two methods; (*a*) by difference between the mucosal transfer and the serosal transfer and (*b*) by direct determination of the amount in the gut wall at

TABLE 4. Transfer of propionate and fluid by everted sac of rat jejunum. The initial weight of the sac was 1·94 g. Duration of experiment 60 min

Propionate (μmole)	
Mucosal transfer	92
Serosal transfer	54
Gut uptake as calculated	38
Gut uptake as estimated directly	42
Fluid (ml.)	
Mucosal transfer	3·01
Serosal transfer	2·34
Gut uptake	0·67
Initial gut fluid content	1·29
Final gut fluid content	1·96
Propionate concn. (mM)	
Initial mucosal and serosal	10·0
Final mucosal	7·2
Final serosal	15·8
Final concn. gradient	8·6
Mucosal concn. transferred	30·6
Serosal concn. transferred	23·1
Final gut propionate concn.:	
from gut uptake as calculated	19·4
from gut uptake estimated directly	21·4

TABLE 5. Comparison of final gut propionate concn. with the serosal concn. transferred and the final serosal concn. The initial mucosal and serosal concn. were the same in each experiment. The figures given are average values for the number of experiments shown. Duration of experiments 60 min

Initial propionate concn. (mM)	Number of experiments	Mucosal concn. transferred (mM)	Serosal concn. transferred (mM)	Final gut concn. (mM)	Final serosal concn. (mM)
5	6	17·4	15·1	9·4	9·2
10	6	31·5	26·3	18·7	17·2
20	7	57·9	43·2	35·6	28·9
46	3	99·5	68·1	56·6	53·4

the end of the experiment. Table 5 shows results obtained from a number of experiments, in which the amount of propionate in the gut wall was calculated from the difference between mucosal and serosal transfer. From these results it is seen that (1) there is good agreement between the values obtained for gut uptake as calculated and as obtained by direct determination; (2) the mucosal concentration transferred was always greater than the serosal concentration transferred, and (3) the final gut concentration was always intermediate between the serosal concentration

transferred and the final serosal concentration. The following interpretation of these results is offered. The fluid and solutes which are transferred by the epithelial cells first pass into the subepithelial tissues. When these can hold no more fluid, the excess spills over into the sac contents. Since the gut wall contains initially fluid but no fatty acid, the propionate concentration in the gut wall must be less than the mucosal concentration transferred, although it will presumably increase during the course of the experiment. If the serosal transfer was only a spill over of fluid from the gut wall, the concentration in the fluid being transferred at any instant could never exceed the concentration in the gut wall, and the average serosal concentration transferred, i.e. what is measured, would probably be less than and could not be greater than the final gut concentration. In fact the final gut concentration was found to be less than the serosal concentration transferred, and this shows that in addition to the spill over there is diffusion of propionate from the higher concentration in the gut wall to the lower concentration inside the sac, but there has been insufficient time to achieve equilibrium. The importance of this interpretation is to stress that the serosal transfer represents only one fraction of what is transferred by the epithelial cells, the rest being in the gut wall. It therefore seems preferable to take mucosal transfer as a measure of transfer capacity rather than serosal transfer.

Effect of variations in fatty acid concentration

The transfer of acetate was studied in concentrations from 5 to 80 mM and the results are seen in Figs. 1–3. Figure 1 shows the mucosal transfer of acetate and water. Acetate transfer increases with increase in initial concentration up to 60 mM, and beyond this point there is reduction in mucosal transfer. In contrast, water transfer remains fairly constant up to a concentration of 40 mM, but at 60 mM there is some reduction in transfer, and at 80 mM a very considerable reduction. Higher concentrations of acetate evidently have an inhibitory effect on transfer of both acetate and fluid. The relation between water transfer and acetate transfer is not, however, direct and at least some independent variation of acetate and water transfer is possible.

Figure 2 shows the mucosal and serosal acetate concentrations transferred, i.e. the concentration of acetate in the fluid leaving the mucosal solution and the concentration in the fluid entering the serosal side. The straight line in Fig. 2 shows what would happen if the concentration transferred was equal to initial mucosal and serosal concentrations. Up to an initial concentration of 60 mM the concentration of acetate leaving the mucosal fluid is greater than the concentration entering the serosal fluid, and both are greater than the initial mucosal and serosal concentration.

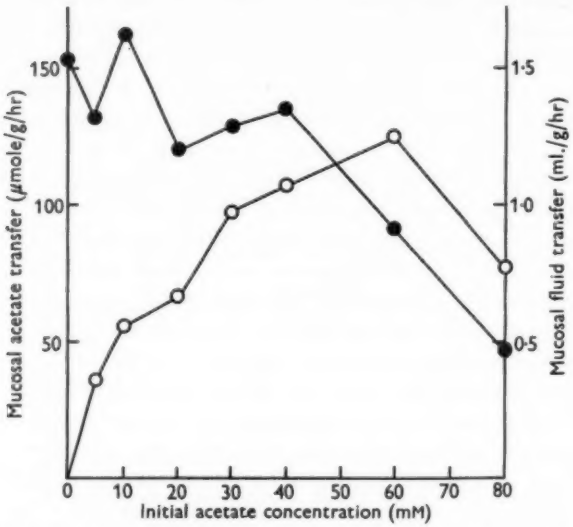

Fig. 1. Effect of initial acetate concentration on mucosal fluid transfer (●) and mucosal acetate transfer (○).

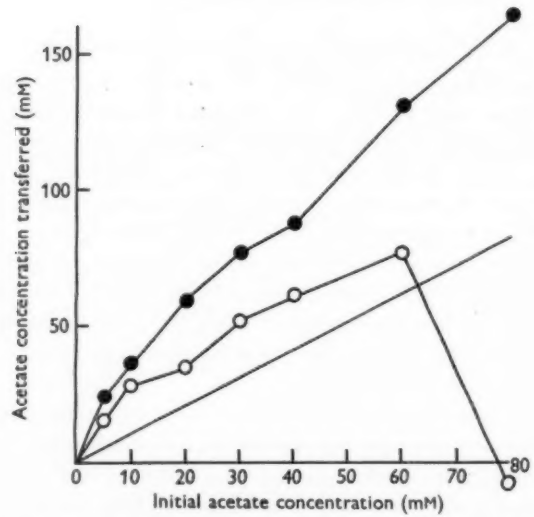

Fig. 2. Effect of initial acetate concentration on the mucosal acetate concentration transferred (●) and serosal acetate concentration transferred (○). The straight line through the origin indicates what would happen if the concentration transferred was equal to the initial concentration.

Thus, up to 60 mM there is active movement of acetate in the sense that concentration of acetate is taking place. At 80 mM there is a dramatic change in the serosal concentration transferred, and this becomes negative. At this concentration acetate moves into the tissue from the serosal fluid, although some fluid is transferred in the reverse direction, i.e. from the intestinal wall into the serosal fluid. The mucosal concentration transferred, however, continues to increase in spite of evidence of inhibition of transfer at high concentrations. Thus a high mucosal concentration transferred is not proof of active concentration of acetate by the intestine. The reason for this is fairly obvious. Initially there is no acetate in the intestinal wall and presumably acetate will diffuse in from both sides. If this

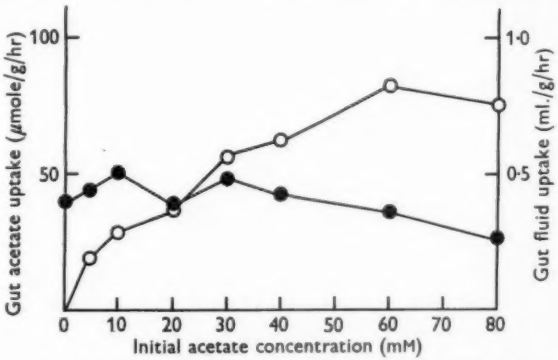

Fig. 3. Effect of initial acetate concentration on gut fluid uptake (●) and gut acetate uptake (○).

is accompanied by only very small fluid movement, as happens with 80 mM acetate, the mucosal concentration transferred may be very high. In contrast, if the serosal concentration transferred is higher than the initial mucosal and serosal concentrations, this is good evidence of concentration of a substance by the intestine.

Figure 3 illustrates the acetate and fluid uptake by the gut and shows that up to an initial concentration of 60 mM the fluid uptake by the intestine remains fairly constant, while the gut acetate uptake is roughly proportional to the initial concentration. Comparison of Fig. 1 with Fig. 3 shows that at these concentrations the gut wall retains about one third of the fluid transferred from the mucosal side, and slightly more than one half of the acetate. The figures are given in Table 6. The proportion of water and acetate retained in the tissues remains remarkably constant in each case until a high initial concentration of acetate (80 mM) is used, when the proportion retained rises sharply. At this stage the amount of both acetate and fluid passing into the sac is diminished much more than

the amount retained by the intestine, and this is shown in Table 6 by an increase in the value of the fraction of mucosal transfer retained in the tissue.

TABLE 6. The fraction of mucosal transfer of acetate and water retained in the gut wall at different initial acetate concn. The figures are average values for a number of experiments. Duration of experiments 60 min

Initial acetate concn. (mM)	Number of experiments	Fraction of mucosal transfer retained in gut wall	
		Fluid	Acetate
5·2	5	0·33	0·59
10·1	3	0·31	0·50
20·2	13	0·32	0·55
30·1	5	0·37	0·57
40·8	10	0·31	0·52
57·5	6	0·36	0·65
80·0	3	0·66	1·00

Michaelis–Menten kinetics

If a substance is transferred by an active process one possibility is that at least one stage in the process may be the combination of the substance with a carrier or an enzyme, and if this stage is rate-limiting then the kinetics of the whole process might conform to the type described by Michaelis & Menten (1913). In testing this the initial concentration and the rate of mucosal transfer have been used. Since there is obvious evidence of inhibition at 80 mM, values at this concentration were omitted from consideration. One of the standard methods of investigating Michaelis–Menten kinetics is that used by Lineweaver & Burk (1934) in which the reciprocals of the concentration and rate are plotted. A weighted regression analysis of the reciprocals of concentration and rate gave a regression equation:

$$y = 0{\cdot}199x + 0{\cdot}0044,$$

where y is the reciprocal of the rate (expressed as μmole/g wet wt. tissue/hr) and x of the concentration (expressed as mM). The intercept on the abscissa was $-0{\cdot}022$, and the negative reciprocal of this (Michaelis constant) is 45 mM. The 95% confidence limits of this value were 37–57 mM. Tests for curvilinearity did not suggest that a straight line was not the best fit for the experimental points.

Transfer of other fatty acids

Experiments were carried out with different concentrations of the other fatty acids, and the mucosal and serosal transfers *in vitro* are shown in Fig. 4. This shows (1) that inhibitory effects are produced by all the fatty acids tested, (2) that the concentration necessary to cause inhibition

decreases with increasing length of chain, (3) that there is no obvious difference in the rate of transfer of odd and even members of the series, and (4), that the inhibitory effects are much more marked on serosal transfer than on mucosal transfer. When mucosal transfer is reduced, a larger fraction of the total serosal transfer is retained in the gut wall, so that what spills over into the sac contents (serosal transfer) is reduced much more, relatively, than the mucosal transfer.

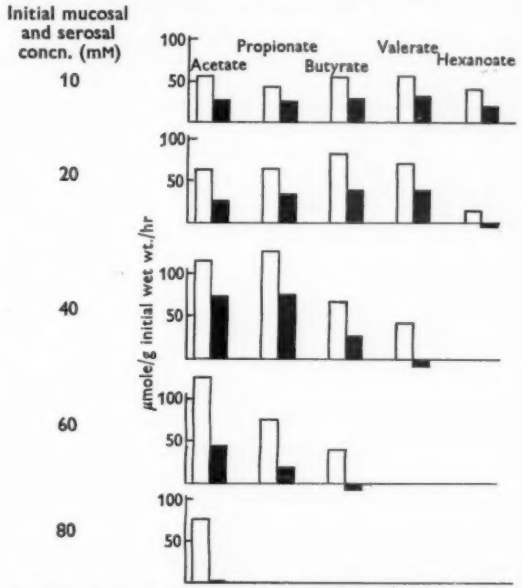

Fig. 4. Relation between length of carbon chain and transfer of fatty acid at various concentrations. The mucosal transfer is shown by white rectangles and the serosal transfer by black rectangles (μmole/g initial wet wt./hr). The lengths of carbon chain were 2 (acetate), 3 (propionate), 4 (butyrate), 5 (*n*-valerate) and 6 (*n*-hexanoate). Each vertical row of mucosal and serosal transfers gives the values for the fatty acid indicated above the top line.

The inhibitory effect of high concentrations of fatty acids is seen also on the final concentration gradient; and this is shown in Tables 7 and 8. Table 7 gives detailed figures for acetate, including the final mucosal and final serosal concentrations. At low concentrations, i.e. up to 40 mM, the increase in serosal concentration is greater than the decrease in mucosal, owing presumably to the much greater volume of fluid on the mucosal side. At 60 mM the increase in serosal is about equal to the decrease in mucosal, suggesting that a greater proportion of the mucosal transfer is being accumulated in the gut wall, but there is still a positive concentration gradient. At 80 mM the inhibition is obvious, with acetate entering the

gut wall from both mucosal and serosal fluids. The final serosal concentration is less than the initial serosal concentration, and the final concentration gradient is negative. Table 8 gives in summary the results with all the fatty acids, the figures for acetate being included for comparison with the others. Evidence of inhibition at high concentrations is seen in the decrease and eventual reversal of final concentration gradient, and the concentration necessary to cause inhibition decreases with increasing length of carbon chain. This negative concentration gradient has been

TABLE 7. Final concn. of acetate at different initial concn. The figures are average values from a number of experiments. The last column gives the final concn. gradient, which is regarded as positive when the final serosal concn. is higher than the final mucosal concn. Duration of experiments 60 min

	Concentration (mM)			
No. of experiments	Initial mucosal and serosal	Final mucosal	Final serosal	Final concn. gradient (mM)
5	5·2	4·0	8·9	4·9
3	10·1	8·2	17·6	9·4
13	20·2	18·3	25·9	7·6
10	40·8	38·0	48·4	10·4
6	57·5	54·5	61·3	6·8
3	80·0	75·5	70·3	− 5·2

TABLE 8. Effect of initial concn. of fatty acids on the final concn. gradient. The final concn. gradient is the difference between final serosal and final mucosal concn. It is considered positive when the final serosal is greater than the final mucosal. Duration of experiments 60 min

	Final concentration gradient (mM)				
Initial concn. (mM)	Acetate	Propionate	Butyrate	Valerate	Hexanoate
5	4·9	6·0	4·6	5·9	5·9
10	9·4	7·8	8·6	12·5	6·0
20	7·6	12·2	12·3	15·2	−4·7
40	10·4	12·0	5·4	− 8·2	—
60	6·8	0·9	− 8·7	—	—
80	− 5·2	—	—	—	—

obtained by Smyth & Taylor (1958) in studying the effect of 2:4-dinitrophenol on fatty acid transfer, by Parsons *et al.* (1958) in studying the effect of phlorrhizin on glucose transfer, and by Jervis & Smyth (1960) in studying the inhibition of amino-acid transfer. In the absence of an active entry from the mucosal side substrate will probably diffuse into the gut more or less equally from both sides. If complete equilibrium is not achieved during the experimental period the small volume of serosal fluid will result in a greater fall in concentration in the serosal fluid than in the mucosal fluid; and hence a negative final concentration gradient will be obtained.

The inhibitory effects of higher concentrations of fatty acid are observed not only on the transfer of fatty acid, but also on fluid transfer. Details

of this are given in Fig. 5 where it is evident that the concentration necessary to cause inhibition of water transfer also decreased with increasing length of carbon chain.

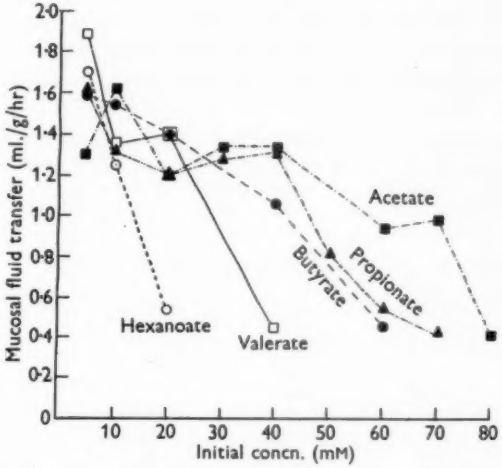

Fig. 5. Effect of initial concentration of a number of fatty acids on the mucosal fluid transfer.

Experiments in vivo

The comparison of rates of absorption of different fatty acids was also studied *in vivo*, by comparing the amounts of fatty acid disappearing from the intestine of the anaesthetized rat in two successive periods in one animal. In each period 50 ml. saline containing fatty acid was circulated through the intestine. In some experiments acetate was present in the intestine during both periods; in other experiments acetate was present during one period, but a different fatty acid was present during the other period. The results are shown in Table 9. This gives the total amount of fatty acid absorbed during the first period and during the second period. The third column of figures shows the ratio of the amount of fatty acid absorbed in the second period to that absorbed in the first period. When acetate was used for both periods the amount absorbed in the second was about 90 % of that in the first. A similar figure for glucose was found by Jervis *et al.* (1956) and for some amino acids by Jervis & Smyth (1959*a*). The last column gives the relative rate of the fatty acid other than acetate, compared with the rate for acetate. It is calculated by assuming that a rate during the second period of 90 % of that of the first period represents equal capacity for absorption for the two fatty acids. The rate for acetate is then considered to be 1, and the figure in the last column is the rate

relative to this of the other fatty acid studied. For example, in the fifth line of the table 365 μmole acetate was absorbed during the first period and 389 μmole propionate during the second period. If acetate had been present in the second period it is assumed that the rate would have been 90% of 365, i.e. 329 μmole. The ratio 389:329 = 1·18:1, and this figure, given in the last column is therefore a measure of the relative rate of absorption of propionate as compared with acetate. In the seventh line

TABLE 9. Rates of absorption *in vivo* of various fatty acids compared with absorption of acetate. In all cases the initial concn. of fatty acid was 20 mM, and the initial volume 50 ml., the duration of the first and second periods 20 min and the interval between the two periods 20 min. For explanation of the relative rate in the last column see text.

First period		Second period			
Fatty acid	Amount absorbed (μmole) a	Fatty acid	Amount absorbed (μmole) b	$\frac{b}{a} \times 100$	Relative rate of absorption compared with acetate
Acetate	412	Acetate	380	92·2	—
Acetate	452	Acetate	381	84·2	—
Acetate	433	Acetate	396	91·5	—
Acetate	461	Acetate	417	90·5	—
Acetate	365	Propionate	389	100·6	1·18
Acetate	424	Propionate	393	93·0	1·03
Propionate	512	Acetate	434	85·0	1·06
Acetate	537	Butyrate	480	89·2	0·99
Acetate	466	Butyrate	480	102·3	1·14
Butyrate	530	Acetate	414	77·8	1·15
Acetate	432	Valerate	416	96·4	1·07
Acetate	442	Valerate	466	105·5	1·17
Valerate	486	Acetate	382	78·5	1·15
Valerate	455	Acetate	362	79·5	1·13
Acetate	428	Hexanoate	462	107·9	1·20
Hexanoate	557	Acetate	408	73·4	1·23

of the table 512 μmole propionate was absorbed during the first period and 434 μmole acetate during the second. It is assumed that if acetate had been present during the first period the amount absorbed would have been 482 μmole. The ratio 512:482 = 1·06:1. This method can only give approximate comparisons, as it ignores the possible effect of the absorption of one fatty acid on that of a different fatty acid in a subsequent period. None the less, the method offers a relatively simple way of making comparisons and might certainly be expected to show differences between the odd and even members of the series if large differences existed. The results suggest that propionate, butyrate, valerate and hexanoate may all be absorbed at a slightly faster rate than acetate, but there is no evidence of a difference between the odd and even members of the series.

It should be noted that there are important differences between the experiments *in vitro* and *in vivo*. In the former any transfer which occurs

must take place against a concentration gradient, but in the latter this is not the case, and the amount transferred *in vivo* might be expected to be much greater, depending on how much acetate is transferred by diffusion in addition to movement by an active process. Exact comparisons of rates *in vivo* and *in vitro* are not possible, but a rough comparison can be made from the figures in Table 3 and Table 9. In both cases the initial concentration of acetate is 20 mM. In the experiment *in vitro* in Table 3 the mucosal transfer by a sac 30 cm long was 161 μmole/hr. In the experiments *in vivo* in Table 9 the average rate of disappearance of acetate from the intestine was 440 μmole/20 min for the whole jejunum and ileum. The 30 cm sac represents something less than half of the intestine. If the intestine transfers equally well in all parts, the transfer *in vivo* is about four times that *in vitro*. Preliminary experiments at present being carried out suggest that the ileum transfers less well than the jejunum, so that the difference between the rates *in vivo* and *in vitro* might well be greater than this. In the absence of further information it is premature to speculate how much of the difference between the rates *in vivo* and *in vitro* is explained by diffusion, and how much by the different conditions *in vivo* and *in vitro*. One important difference is that the inhibition of fatty acid transfer observed with 20 mM hexanoate *in vitro* is not observed *in vivo*. This aspect of the problem is being further investigated.

DISCUSSION

The experiments described give a more complete picture of the transfers of lower fatty acids than has hitherto been available, and their relation to water transfer. They confirm previous results that the isolated intestine is able to transfer lower fatty acids against a concentration gradient, and they extend the previous observations to include also valeric and hexanoic acids. Use of a more extensive range of fatty acids makes it possible to compare the transfer of odd and even chain fatty acids. Deuel, Hallman & Reifman (1941) suggested a difference in the rate of intestinal absorption of odd and even members of the series, but this has not been confirmed in the present experiments. Instead it has been found that there is a difference *in vitro* depending on the absolute length of the carbon chain. With increase in concentration of fatty acid an inhibitory effect is produced on transfer of both fluid and fatty acid, and the concentration necessary to produce this effect becomes smaller with increasing length of carbon chain. It should be pointed out, however, that the experimental conditions are very different from those used by Deuel *et al.* (1941). These authors fed the fatty acids by mouth to unanaesthetized rats, and the gastric emptying time could play an important part in the results. While the intestinal concentrations were not known, the total amounts of fatty

acid given were about twice the amount used in our experiments *in vivo*. Comparison of the rates of absorption shows that these were of the same order, e.g. Deuel *et al.* (1941) found an average rate of acetate absorption of 62 mg/hr while our figure *in vivo* was 78 mg/hr. The absorptive activity of the intestine is therefore comparable in the two sets of experiments. A possible explanation of the different results is the duration of the experiments. Those of Deuel *et al.* (1941) lasted 1 hr, and it is possible that different rates of removal of odd and even fatty acids from the blood stream by metabolism might cause different rates of absorption. Our experiments lasted only 20 min, and differences in blood concentration might therefore be much smaller. As regards the actual absorption mechanism, differences in surface tension, fat solubility or dissociation of these particular fatty acids are not related to oddness or evenness of the chain, and these are the properties which might conceivably influence absorption rate.

The mechanism of transfer of fatty acids still remains obscure. Smyth & Taylor (1958) suggested the possibility of a common factor in transfer of fatty acid and water, the possible common factor being transfer of sodium. They pointed out however that the effect of DNP and phlorrhizin showed that inhibition of water and fatty acid transfer did not run parallel. In the present experiments the transfer of water and fatty acid also did not necessarily run parallel and could vary independently within certain limits. On the other hand high concentrations of fatty acid inhibited both fatty acid and water transfer, so that there may be some interrelation of the movement of water and fatty acid. One possibility is passive transfer of fatty acid in an active water stream in addition to active transfer of acetate. A similar possibility has been suggested by Smyth & Taylor (1957) for glucose.

The experiments with different concentrations of acetate suggest the existence of a mechanism which can be saturated. It is of some interest to compare the value of the Michaelis constant with those obtained for other substances. Fisher & Parsons (1953*b*) obtained a value of 8–9 mM for glucose absorption *in vitro* and Riklis & Quastel (1958) obtained a value of 7 mM. Jervis & Smyth (1959*b*) obtained values of 10 mM for L-methionine and values of 19–40 mM for D-methionine and D- and L-histidine, and suggested that D-methionine and D- and L-histidine had a low affinity for the transfer mechanism. The value obtained here for acetate would put it also in the class of substances with a low affinity for the transfer mechanism. The interpretation of Michaelis–Menten kinetics in absorption studies requires considerable care. Jervis & Smyth (1959*b*) have pointed out that there may be very large errors in the determination of the Michaelis constant, and apart from this, various authors, e.g. Fisher & Parsons (1953*b*) and Widdas (1952), have stressed that a number of different kinds of

processes may show Michaelis–Menten kinetics. Apart from the question what kinds of processes exhibit Michaelis–Menten kinetics there is the possibility that experimental results obtained with a certain experimental error may suggest kinetic relationships resembling Michaelis–Menten form. The statistical procedures used show that the assumption of a straight-line relationship between the reciprocals of rate and concentration is consistent with the experimental data, but other kinetic relationships approximating only within certain limits to Michaelis–Menten kinetics could permit a similar conclusion. It would be safe to conclude that the results are not inconsistent with the concept of formation of a carrier or participation of an enzyme process, but it would be unwise to go further than this.

Another possibility is that suggested by Brodie & Hogben (1957) that the movement of acetate across the intestinal membrane may depend on differences in pH on the two sides of the membranes. If the un-ionized part of the molecule is in equilibrium on both sides of the membrane, difference in dissociation would lead to different concentrations of fatty acid. According to this view production of pH changes could be an important factor in fatty acid transfer. Since the presence of glucose leads to different pH values on the mucosal and serosal sides (Wilson, 1954) this would provide a possible explanation for the findings of Smyth & Taylor (1958) on the effect of glucose and inhibitors of glucose transfer on fatty acid transfer. It is even possible that the kinetic relationship observed with different concentrations of fatty acids might be related to the effect of these on glucose uptake and lactic acid production. Our experiments do not test this hypothesis, but further experiments are being designed to do so.

The results provide some basis for discussing the interpretation of experiments *in vitro*, and we envisage the following processes taking place. Fluid and solutes are taken up by the epithelial cells from the mucosal fluid, and this probably corresponds to the first process in physiological absorption. The fluid and solutes are then transferred into the subepithelial spaces, and would *in vivo* be removed by the blood or lymph. Removal by the blood stream cannot occur *in vitro* and the fluid therefore accumulates in the tissue spaces in the gut wall. When no more fluid can be accumulated some spills over into the sac contents. In addition, diffusion of solute also occurs from the higher concentration in the gut wall to the lower concentration in the serosal fluid. It seems to us therefore that the process most nearly related to physiological absorption is mucosal transfer either of fluid or of solutes, and that serosal transfer represents only a fraction of this. This fraction is different for different substances, e.g. the figures for Table 4 show that it is 54/92 or 0·59 for propionate and

2·34/3·01 or 0·78 for water. Furthermore, the fraction is not constant even for one substance, as is shown in Table 6. If the activity of the epithelial cells is to be measured by the change in the serosal side, then the introduction of a term 'total serosal transfer' would be necessary, which would include the increase in the sac contents and in the sac wall. It is perhaps useful to relate our terminology to that used by other authors. What has been called in this paper 'serosal transfer' as distinct from 'total serosal transfer', is equivalent to what was termed 'absorption' by Darlington & Quastel (1953) and Wilson (1956*a*); '$Q_{\text{substrate transferred}}$' by Wilson & Wiseman (1954) and Wilson (1956*b*); 'transference' by Wiseman (1955), 'transfer' by Agar, Hird & Sidhu (1953); and 'transport' by Wilson (1956*c*), and Crane & Wilson (1958). According to the concept put forward here these terms all refer only to an unknown fraction of what is transferred through the mucosal cells.

While mucosal transfer is usually preferable to serosal transfer as an index of absorption capacity, there are some important aspects of serosal measurements, and these apply particularly to concentration changes. In cases of small fluid transfer a high mucosal concentration transferred does not necessarily indicate active concentrating capacity, whereas a high serosal concentration transferred does so. The advantage of serosal concentration transferred over final serosal concentration is that it is a more sensitive index, since the final serosal concentration is an average value dependent on the serosal concentration transferred and the initial serosal concentration. Wilson & Wiseman (1954) first used the everted sac to study movement against a concentration gradient. The concept of mucosal transfer and its relation to physiological absorption extend the usefulness of the everted sac, by making it possible to measure how much of a substance can be moved against a particular concentration gradient.

SUMMARY

1. Experiments have been carried out both *in vivo* and *in vitro* on the transfer by the rat small intestine of acetate, propionate, butyrate, valerate and hexanoate.

2. All these fatty acids can be transferred *in vitro* against a concentration gradient.

3. There is no difference between the odd and even members of the series as regards rate of transfer, either *in vivo* or *in vitro*.

4. Higher concentrations of all the fatty acids have an inhibitory effect *in vitro* on transfer of fatty acid and fluid and the concentration necessary to cause this inhibition decreases with increasing length of chain.

5. The significance of mucosal and serosal transfers *in vitro* is discussed and their relation to physiological absorption.

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MODULATION THRESHOLDS FOR SINUSOIDAL LIGHT DISTRIBUTIONS ON THE RETINA

BY G. WESTHEIMER

From the Ohio State University, Columbus 10, *Ohio, U.S.A.*

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In an optical system, the 'contrast transfer function' describes the relationship between the contrast of test objects whose intensity varies sinusoidally with distance and the contrast of their corresponding images at various spatial frequencies (lines/unit distance). It conveys more information about the characteristics of the system than does the specification of only the resolving power. This approach, widely applied to electrical systems, is strengthened by the formal identity of the transformation equations in diffraction theory between objects and images with those of Fourier analysis and synthesis (Hopkins, 1953). The method can also be applied to the response of a receiving layer, such as photographic film. When a sequence of optical and receiving systems is used in series, the total response of the whole system is deducible from those of its components, provided the principle of linearity holds.

In a physical system when carrying out a sine-wave analysis, one determines the output magnitude for a constant input at various frequencies, but this is not readily feasible in the case of the visual system. Instead, one may reverse the procedure and measure the input required for a constant output, for example, a threshold response. In the present paper data will be presented of the application of such an approach to the retinal and central stages of the visual process, excluding the purely optical ones of the eye.

METHODS

Sinusoidal fringes were created on the retina by a method based on Young's double-slit interference experiment, and this pattern was used to modulate a field of uniform retinal illumination. Light from a compact filament 6 V, 2·75 A lamp (V) is brought to a focus in the plane of a narrow slit, S (Fig. 1*a*). After being collimated by lens L_1 the beam from the slit passes through IF, an interference filter ($\lambda_{max.} = 555\,m\mu$), and RR, a 2×2 in. (5×5 cm) Ronchi ruling (150 lines/in.; 60 lines/cm). The diffraction image is brought to a focus in plane P_1 by lens L_3. Here is placed a mask that blocks out all but the zero-order and the two first-order images of the diffraction pattern. The zero-order is passed through a strip of polaroid and the two first-order images are passed through pieces of polaroid cut in a direction perpendicular to that for the first-order image. These beams are then focused in the pupil of the subject's eye by lens L_4. A single sheet of analysing polaroid, A, is also

placed in the beam. When its direction is parallel to that of the polaroid in the zero-order beam, only the latter enters the eye. It produces a field of uniform retinal illumination (Fig. 1*b*). When the analyser is now rotated through 90°, only the two first-order beams are imaged in the pupil. They constitute two coherent images which give rise to Young's interference fringes on the retina unaffected by defocusing of the optics of the eye or by aberrations in the usual sense.

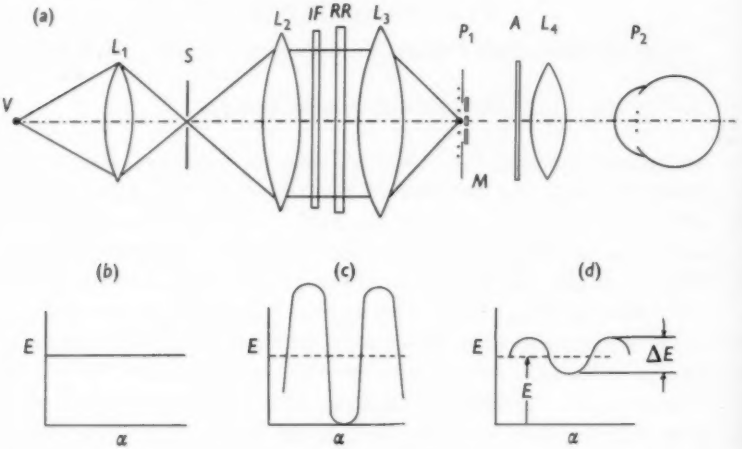

Fig. 1. (*a*) Schematic diagram to illustrate optical arrangement used to create interference fringes on the retina. A slit S is illuminated by light from a lamp V focused by lens L_1. A collimated beam from the slit passes through IF, an interference filter, and RR, a Ronchi ruling with 150 lines/in. (60 lines/cm). The diffraction pattern is imaged by lens L_2 in P_1. A mask M screens all but the zero- and the two first-order diffraction images. The zero-order image passes through a piece of polaroid and the two first-order images pass through other pieces of polaroid at right angles to the former. The pattern in P_1 is imaged by lens L_4 in P_2, the plane of the pupil of the subject's eye. An analysing polaroid A permits the presentation of the zero-order image only, or of the two first-order images only, or of both in proportions according to the $\sin^2$ law. (*b*) Uniform intensity distribution on retina when only the zero-order image is transmitted by analyser. (*c*) Sinusoidal intensity distribution when only the two first-order images are transmitted. (*d*) Modulated sinusoidal distribution in an intermediate position of the analysing polaroid. The normalized ratio $\bar{E}/\Delta E$ at threshold visibility for a given spatial frequency was used to define the modulation sensitivity at that frequency.

The intensity distribution on the retina is then sinusoidal (Fig. 1*c*). Expressed in quantities applying to the object space of the eye, the visual angle corresponding to the separation of two successive maxima is given by the equation

$$\Delta\alpha = \lambda/2a, \qquad (1)$$

where λ is the wave-length in air of the light used, and $2a$ the separation of the two first-order images in the pupil. When expressed in this way, $\Delta\alpha$ is a visual angle in air and is measured in radians.

If the light flux in the zero-order image is equal to the total flux in the two first-order

images, $E(\alpha)$, the distribution of illumination across the retina as a function of the visual angle α, is given by

$$E(\alpha) = E\cos^2\theta + E\sin^2\theta\,(\cos(2\pi\alpha/\Delta\alpha)+1), \qquad (2)$$

where θ is the orientation of the analysing polaroid with respect to that of the polaroid through which the zero-order beam is passing. When $\theta = 0$, $E(\alpha)$ is a constant, E. When $\theta = \frac{1}{2}\pi$, $E(\alpha) = E(\cos(2\pi\alpha/\Delta\alpha)+1)$, i.e. the retinal illumination is a sinusoidal function of frequency $(\Delta\alpha)^{-1}$ and has crests of height $2E$ and troughs of zero. By varying θ within the limits of 0 and $\frac{1}{2}\pi$, one can obtain any modulation of the amplitude of the sinusoidal image distribution, the mean retinal illumination level $\bar{E}$ remaining constant. While the assumption of 100 % modulation at $\theta = \frac{1}{2}\pi$ is somewhat idealized, because it neglects to take into account possible scattering of the beams within the eye, the actual distribution is not likely to differ from this by more than a few per cent.

The subject places his eye so that the image of P_1, as formed by lens L_4, falls in the pupil of his eye. He sees the available area of L_4 filled with monochromatic light. When $\theta = \frac{1}{2}\pi$, the area is traversed by stripes and as the polaroid is rotated towards $\theta = 0$ the fringes gradually become diluted by the uniform field that is seen as θ approaches zero. In the apparatus the total flux in the two first-order images falls a few per cent short of that in the zero-order image. This difference, accurately determined by placing a photomultiplier in the plane usually occupied by the pupil of the eye, is taken into account in the computation by which the modulation is obtained from the angle of the analyser at threshold.

The spatial frequency, i.e. the number of complete sinusoidal intensity cycles per unit distance along the retina, can be varied by changing the position of the lens L_4 and the plane of the pupil to produce images with several different magnifications of the pattern in P_1 in the subject's pupil. The relationship between the spatial frequency $(1/\Delta\alpha)$ and the separation $2a$ of the two first-order images in the pupil is given by equation (1). The current in lamp V can be varied to control the retinal illumination of the field seen in lens L_4. During most of the experiments the retinal illumination was held constant at a level determined by visual match to be equivalent to that of a field of about 30 cd/m^2 seen by an eye with a 5 mm entrance pupil (2200 td). Changes in the light flux may also be produced by varying the slit width of S. This cannot, however, be done indiscriminately, since the slit width affects the coherence of the radiation in the two first-order images, interference of which is the basis of the fringe pattern on the retina. In fact, an increase in the slit width decreases the visibility of the fringes and this is an alternative method of producing gradual dilution of the pattern. In this study a slit width was used that was determined experimentally to produce effectively coherent images. This was done by finding the angle θ for extinction of the fringes as a function of slit width. At each spatial frequency a slit width was used that is well within the range of independence of threshold dilution with respect to slit width.

Procedure. The subject had one eye occluded and placed his other eye, without any spectacle correction, so that the image of the pattern in P_1 fell accurately in the centre of his pupil. He then rotated the analysing polaroid from the position $\theta = \frac{1}{2}\pi$ until the fringes could no longer be distinguished. This was repeated five or ten times, depending on the spread of the results, at each spatial frequency used. Finally, the absolute resolution threshold was determined by setting $\theta = \frac{1}{2}\pi$ (100 % modulation) and moving lens L_4 and the subject to increase the spatial frequency until fringes could no longer be distinguished.

In graphing the results, the lead of Schade (1956) was followed in the use of the normalized ratio $\bar{E}/\Delta E$, where $\bar{E}$ is the average level of the retinal illumination and ΔE the peak-to-peak modulation required for threshold at a given spatial frequency (Fig. 1*d*). The ratio will here be called *modulation sensitivity*. It is a measure of the responsiveness of the visual system and is inversely related to the modulation at the threshold of fringe visibility at a given spatial frequency.

RESULTS

Measurements of the minimum modulation required for threshold fringe visibility were obtained at five fringe widths (spatial frequencies) for three normal observers. In addition, the absolute threshold for fringe resolution at 100% modulation was obtained for each observer.

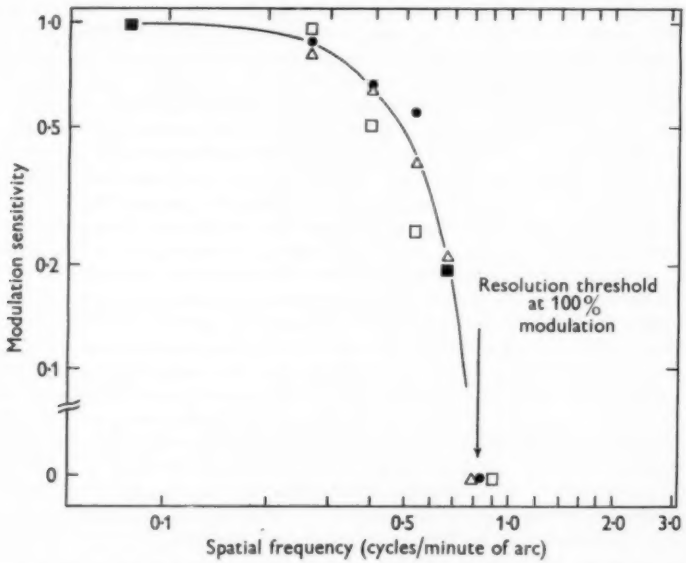

Fig. 2. Average values of modulation sensitivity of three normal observers at various spatial frequencies, normalized at 0·08 cycles/minute of arc, and an averaged curve (log. scales). ●, WI; □, WE; △, KI.

Each subject's results are normalized to make the ratio $\bar{E}/\Delta E$ equal to unity at the lowest frequency used, 0·08 cycles/minute of arc. The normalization factors for the three observers WI, WE and KI are 0·328, 0·329 and 0·270, respectively. The mean modulation sensitivity for each observer at each spatial frequency used is shown in Fig. 2 on logarithmic co-ordinates and an average curve is drawn for all three observers.

It is difficult to increase the illumination level with the apparatus used because it is wasteful of light. By overrunning the lamp for short periods, however, it was possible to test whether any important changes occur in the modulation sensitivity curve as a function of retinal illumination in the photopic region. No significant changes were found in the range of about 1 $\log_{10}$ unit above and below the level used, i.e. in the range between about 200 and about 20,000 td. This is not unexpected since such other

psycho-physical thresholds as visual acuity and brightness discrimination remain essentially unchanged in this region.

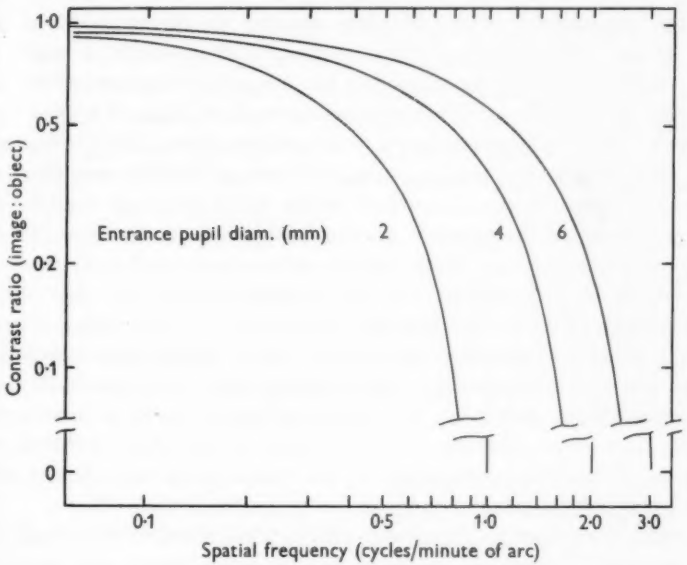

Fig. 3. Contrast transfer functions (ratios of contrasts in images and corresponding objects for sinusoidal light distributions) of optical system like that of the human eye but free from aberrations with round pupil of various diameters in monochromatic light of wave-length $\lambda = 555$ mμ (log. scales).

DISCUSSION

Figure 3 shows for various pupil sizes, for an optical system like the eye's but free from aberration, the loss of contrast between object and image for an object made of a series of bright and dark lines, the light intensity varying sinusoidally from one line to the next (Hopkins, 1953). When the lines are far apart, diffraction has little effect on reducing contrast, but as they are brought closer together contrast is lost relatively quickly. When the lines are separated by λ/D radians, where D is the diameter of the eye's pupil, diffraction has spread each out in such a way that the light distribution on the retina is uniform and resolution is impossible, irrespective of whether the receptive layer is capable of it.

The experiments here described show that, if one produces across the retina a similar series of bright and dark lines with sinusoidal variation in intensity and varies their contrast, quite suddenly a much bigger contrast is needed for the lines to be seen. There is similarity between the

experimentally measured retinal modulation sensitivity curve (Fig. 2) and the theoretical contrast transfer function for an ideal optical system like the eye's with a 2 mm round pupil (Fig. 3).

In the experiment, when the line spacing at the retina is reduced below 34–38 seconds of arc for the width of a single dark or bright line, i.e. beyond 0·80–0·87 cycles/minute of arc, resolution is no longer achieved, even with 100 % contrast. This measurement of the limit of spatial resolution of the visual system, obtained by a method substantially independent of the optical system of the eye, is in good accord with the results of most previous comparable experiments (O'Brien, 1951), although occasionally a study will report a threshold of grating visibility at a significantly higher frequency (Byram, 1944). There was no evidence in any of the three subjects of the effects described by Byram, who claimed to have seen short crooked line segments at very high spatial frequencies ($> 1{\cdot}3$ cycles/minute of arc). It must be admitted that when using interference methods, as Byram did, it is difficult to avoid creating pattern components due to extraneous effects. With 100 % modulation the subjects in this study all saw fine dark lines, restricted to the region of the point fixated, which disappeared into the background as the spatial frequency was increased beyond the threshold.

Previous investigators (Hartridge, 1918, 1922; Shlaer, 1937) could make only tentative statements, based on computation, about the retinal light distribution at the threshold of resolution, particularly about the depth of the minimum between two adjacent maxima just distinguishable as separate. The present study affords a quantitative description, based on direct measurement with a repetitive pattern, of the minimum required at the retina for resolution threshold when adjacent maxima are separated by various distances. The results generally confirm the conclusion that ordinarily the limit of visual resolution is set by the retina or visual pathway, even though the optical system of the eye (Fig. 3) can potentially allow a better performance when the pupil diameter exceeds about 2 mm.

The visual response to illumination gradients (e.g. Mach bands) suggests that there might be somewhere in the visual system a spatial 'high pass filter', i.e. that the response to low frequencies might be relatively attenuated. Schade (1956) gives response curves for the whole visual system, including the optics of the eye, showing such low-frequency attenuation dependent on the luminance level, although his basic data are not fully presented. In the present study no such attenuation was demonstrated in the photopic region over the range of spatial frequencies covered. (See Fig. 2; it was ascertained by check tests, not shown in the figure, that the curve is monotonically decreasing.)

It might appear to be a direct step from the results shown in Fig. 2 to the prediction by means of Fourier synthesis of responses to light distributions more complex than sinusoidal ones. In this kind of approach one would multiply each Fourier component of the light distribution on the retina with a weighting factor—a function of spatial frequency—which defines the response characteristics of the visual system. Fourier synthesis would then permit the prediction of the final visual response—the apparent brightness distribution in the visual field. But the modulation sensitivity curve shown in Fig. 2 was obtained by threshold methods and is, therefore, not necessarily identical with the curve of ratios of image and object contrasts for equality of non-threshold response with which one would have to multiply the Fourier components of input light distributions for the purpose of Fourier synthesis. Before extending the application of Fourier methods in this way, it would first be necessary to obtain by direct measurement a curve of the contrasts that are required for equality of non-threshold responses at various spatial frequencies. It would also have to be determined whether the principle of additivity holds in this system.

The results in the present paper are, however, directly applicable to the use of Fourier methods in the analysis of the optical system of the eye. They provide the basis for the evaluation by psycho-physical means of the contrast transfer function of the optics of the eye under various conditions of pupil size, aberrations, defocusing, and so on. Such an approach will permit the specific description of retinal light distributions which is so desirable in experiments in which the intact optics of the eye are used in the investigation of the physiology of the retina and the visual pathway.

SUMMARY

1. By a method based on Young's double-slit interference experiment, a retinal light distribution was created in which intensity varies sinusoidally with distance. This pattern was used to modulate a field of uniform retinal illumination. The average retinal illumination remained constant at 2200 td. Monochromatic light of wave-length 555 mμ was used.

2. The minimum depth of modulation required for visibility of fringes was measured at various spatial frequencies (fringes/minute of arc) in three normal observers.

3. The average modulation sensitivity curve when normalized is similar to the theoretical contrast transfer function of a perfect optical system like the eye's with a round pupil of 2 mm diameter.

4. The values for the absolute threshold for fringe resolution at 100 % modulation in the three observers ranged from 0·80 to 0·87 cycles/minute

of arc, i.e. 34–38 seconds of arc for the width of a single light or dark bar at threshold.

5. The optical arrangement ensured that the retinal illumination pattern remained unaffected by aberrations or defocusing of the optical system of the eye. The measurements were thus functions of only the retinal and central stages of the visual process.

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THE EFFECT OF BRADYKININ, SERUM KALLIKREIN AND OTHER ENDOGENOUS SUBSTANCES ON CAPILLARY PERMEABILITY IN THE GUINEA-PIG

BY K. D. BHOOLA*, J. D. CALLE AND M. SCHACHTER
From the Department of Physiology, University College London

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Experiments with crude preparations of bradykinin have shown that they increase capillary permeability in the guinea-pig, rabbit (Holdstock, Mathias & Schachter, 1957) and man (Herxheimer & Schachter, 1959). The present experiments with highly purified preparations demonstrated that bradykinin is indeed a very potent enhancer of permeability. It was of interest therefore to study also the effect of serum kallikrein on capillary permeability, since this substance occurs normally in an inactive form in the blood of man and animals, and when activated releases kallidin, a polypeptide closely resembling bradykinin (see Frey, Kraut & Werle, 1950; Werle, 1955, 1960). Serum kallikrein was also found to be effective in increasing capillary permeability, and our results suggest that this activity is, in fact, due to the release of kallidin. The effects of serum kallikrein and bradykinin were similar qualitatively and quantitatively and both differed in a number of ways from the effect of histamine.

Since plasmin, the fibrinolytic enzyme in plasma, resembles kallikrein in that it exists in blood in an inactive but activable form (Müllertz, 1956; Biggs & MacFarlane, 1957), and has been found by some workers to be a potent releaser of a substance indistinguishable from bradykinin (Beraldo, 1950; Lewis, 1958), we compared its properties with those of serum kallikrein. Our experiments showed that kallikrein differed from plasmin in several ways. Plasmin was relatively ineffective in increasing capillary permeability and failed to release a smooth-muscle stimulating agent from plasma under conditions in which serum kallikrein was very active; on the other hand, plasmin was far more effective than kallikrein in producing fibrinolysis.

A preliminary communication of some of these results has previously been made to the Physiological Society (Bhoola & Schachter, 1959).

* M.R.C. Scholar.

METHODS

Capillary permeability. Albino guinea-pigs weighing 350–450 g were clipped of their fur and the skin of both flanks was depilated, beginning close to the dorsal mid line and extending 4–6 cm ventrally, between hips and shoulders. The guinea-pigs were injected intravenously with a 5% solution of pontamine sky blue 6BX (E. Gurr) in saline via the saphenous vein (Miles & Miles, 1952) in a dose of 1·2 ml./kg. Intradermal injections of the various test substances in saline were then made in 0·1 ml. volumes with syringes of 0·25 ml. capacity fitted with fine, short-bevelled, intradermal needles ($\frac{1}{2}$ in. (1·3 cm), gauge No. 26). Needles were carefully sharpened on a fine stone before each experiment. The diameter of the blue lesion at the site of injection, due to dye leaving the circulation, was measured 25 min after the intradermal injection. The size of the lesion was taken as the mean of the largest diameter and the one at right angles to it, measurements being made on the unanaesthetized animal. Lesions measuring less than 3 mm are not significant since a mean diameter of this size represents 'traumatic' blueing due to the injury of injection.

Bradykinin, serum kallikrein and histamine were injected in concentrations of 200, 50, 10, 2 and 0·5 μg/ml. in 0·1 ml. volumes, each substance in a row on the flank of the guinea-pig, as is shown in Pl. 1, fig. 1. In order to derive dose–response curves for each substance it was found necessary to vary only the positions of the rows of different substances relative to one another on the flank. Both flanks of the animal were injected in succession in this way, the lesion diameters measured, and dose–response curves obtained, each point on the curve representing the mean diameter of a minimum number of six lesions in at least three animals. This procedure was adopted because of its relative simplicity and because we found in preliminary experiments that complete randomization of different doses of these substances in Latin square distributions on the flank revealed no significant variation in the size of lesions produced by the same concentration of a substance in different locations. This is correct, provided that the region of the flank injected is confined between hip and shoulder, and does not extend ventrally for more than 4 cm from the dorsal mid line. Injections made beyond this limit ventrally produce progressively larger lesions with the same concentration of drug.

To test the effect of the antihistamine drug mepyramine the following procedure was adopted. The various test substances were injected intradermally after the animal had been injected with pontamine blue, and measurements of the lesions were made 25 min later as already described. The animal was then injected with mepyramine (0·1 mg/kg) via the saphenous vein, and intradermal injections of the test substances were repeated on the other flank. Twenty-five minutes later these lesions were measured and compared with those obtained in the same animal before treatment with mepyramine.

Soya-bean trypsin inhibitor (SBTI) and ovomucoid trypsin inhibitor (OI) were incubated for 30 min at room temperature with bradykinin, kallikrein or histamine when tested for their inhibitory activity. In some animals a concentration of SBTI of 200 μg/ml. itself produced an increase in permeability. Animals used for testing were therefore given an initial injection of SBTI to ascertain that they did not react to the drug in the concentration being used.

Fibrinolysis. For measuring fibrinolysis the fibrin plate method described by Astrup & Müllertz (1952) was used, but with some minor modifications which we found necessary to provide a suitable consistency of fibrin clot for quantitative measurements of lysis under our conditions. The fibrin clots were made on Petri dishes of 10 cm diameter by clotting 8 ml. of 0·15% (clottable protein) of purified human fibrinogen solution with 0·17 ml. of bovine thrombin (17 u.). The fibrin plates were incubated for 1 hr at 37° C, a procedure which provided a clot of suitable and constant consistency. A drop of 0·03 ml. of each enzyme solution in veronal buffer was then carefully placed on the fibrin surface from a micropipette. The plate was divided into four compartments and at least eight spots could

be applied to each plate. They were incubated for approximately 16 hr at 37° C and the diameters of each lysed area measured. Inhibitors (SBTI and OI) were made up in the same buffer and mixed with the test solutions for 30 min before applying a drop of the mixture to the fibrin plate in the same way.

Isolated guinea-pig ileum. A loop of ileum was suspended in an 18 ml. bath of Mg-free Tyrode solution containing atropine and mepyramine (10^{-5} g/l.).

Drugs and other materials

Bradykinin was prepared by the action of crystalline trypsin on heated ox serum globulin (Holdstock *et al.* 1957) and purified chromatographically so that it contained 900 u./mg freeze-dried material, in terms of the units of Rocha e Silva, Beraldo & Rosenfeld (1949) when tested on the guinea-pig ileum. This bradykinin preparation was kindly provided by Mr W. A. Jones and Dr I. M. Lockhart.

Serum kallikrein was prepared by the casein adsorption method (Werle, 1936) and purified by fractionation with acetone, alcohol and ammonium sulphate. Our two preparations, kindly provided by Professor E. Werle, contained 3 and 35 kallikrein u./mg protein. The kallikrein solutions, containing a trace of toluol as a preservative, were dialysed against saline for several hours in the cold before experiments. Solutions were kept frozen until required and once thawed were not used again, as activity was found to decline with thawing and re-freezing of the purer preparations. The weights of serum kallikrein are expressed as weights of protein, based on nitrogen determinations.

Plasmin was a freeze-dried preparation made from human plasminogen activated by streptokinase. It was provided by Merck, Sharp and Dohme, Ltd.

Thrombin. Bovine thrombine, approximately 30 u./mg (S. Maw, Ltd.)

Fibrinogen. Purified human fibrinogen, 80–90% clottable protein (Blood Products Laboratory, Lister Institute, Elstree).

Salivary kallikrein was a freeze-dried preparation from human saliva, as described by Holdstock *et al.* (1957).

Trypsin. Crystalline trypsin (Armour), 2640 u./mg.

Serum and plasma. Human blood, for plasma, was obtained by venepuncture and collected in heparin. Guinea-pig serum or plasma (+ heparin) was obtained by cutting the jugular vein, after killing the animal by a blow on the head. Some of these were heated to 56° C for 3 hr.

Veronal buffer was made up according to the method of Astrup & Müllertz (1952) and consisted of 662 ml. 0·1 M sodium diethyl barbiturate mixed with 338 ml. 0·1 M-HCl diluted with 320 ml. distilled water. The pH of this buffer was 7·8 and ionic strength (I) 0·05.

Soya bean and ovomucoid trypsin inhibitors were crystalline preparations (Worthington).

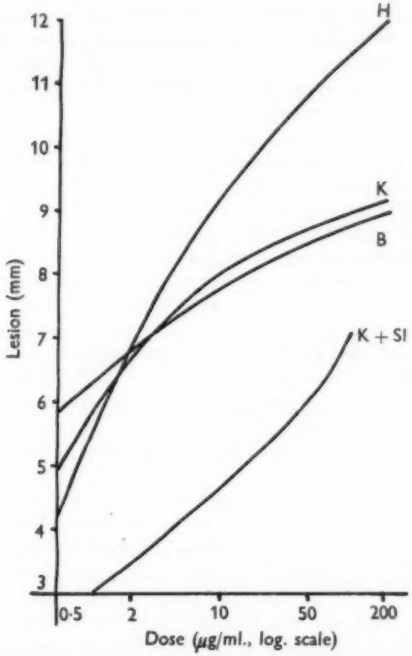
Histamine, atropine and mepyramine were used as acid phosphate, sulphate and maleate respectively; weights of histamine are expressed as base.

RESULTS

Capillary permeability

Comparison of bradykinin, serum kallikrein, histamine and plasmin. Bradykinin and serum kallikrein, when injected intradermally into guinea-pigs with circulating pontamine blue dye, regularly increased capillary permeability and produced lesions of a more intense blue colour than those produced by histamine. In general, increased blueing appeared within 2–3 min of intradermal injection of these substances, whereas the delay was 4–6 min after injection of histamine. Bradykinin and kallikrein,

though still impure, were more effective than histamine in the lower concentrations. The dose–response curves for bradykinin and kallikrein, however, unlike that for histamine, showed a pronounced flattening with the higher concentrations (Pl. 1, fig. 1; Text-fig. 1). The size of the lesion was fully developed in 20–30 min in all instances, and no significant increase was detected after 2 hr.



Text-fig. 1. Dose–response curves of histamine (H), serum kallikrein (K) and bradykinin (B) in increasing capillary permeability in guinea-pigs with circulating pontamine blue dye. 0·1 ml. of each solution was injected intradermally. K + SI, serum kallikrein + soya-bean trypsin inhibitor (200 μg/ml.) incubated at room temperature for 30 min before injection of 0·1 ml. of mixture.

A preparation of human plasmin, which had high fibrinolytic activity, was without effect on capillary permeability. In three animals tested a significant response to intradermal injections began to appear only at concentrations of 500 μg/ml., indicating activity approximately 1000 times less than that of the other substances (Pl. 1, fig. 2).

Effects of inhibitors. Soya bean trypsin inhibitor (SBTI) in a concentration of 200 μg/ml. reduced the permeability-enhancing property of serum kallikrein 50–100 times (Text-fig. 1) on incubation (30 min; room temperature) with solutions of kallikrein containing 0·25–3·0 u./ml., or

less. The effects of higher concentrations of SBTI could not be studied, because in some guinea-pigs a concentration of 200 μg/ml. of SBTI itself produced a significant increase in capillary permeability. Ovomucoid trypsin inhibitor (OI), in concentrations up to 500 μg/ml., failed to affect the permeability-increasing activity of kallikrein under the same conditions. Pre-treatment of the animal with mepyramine (0·1 mg/kg, I.V.) was also without effect (Pl. 1, fig. 3).

The action of bradykinin was unaffected by incubation with SBTI or OI as described above. Also, the dose–response curve of animals pre-treated with mepyramine remained unchanged.

Histamine, like bradykinin, was unaffected by SBTI or OI throughout its activity range, but was practically completely antagonized by pre-treatment of the animal with mepyramine (Pl. 1, fig. 3).

Release of kallidin by serum kallikrein and by plasmin

Serum kallikrein was a potent releaser of kallidin not only from the plasma of the guinea-pig, but also from those of man, ox, dog, cat and rabbit. Heated (56° C, 3 hr) dialysed plasma was used in most experiments (to eliminate the release of kallidin by dilution, and to destroy the inactivators of kallidin and of kallikrein), but similar results were obtained with unheated plasma. We could readily detect the release of kallidin from human or guinea-pig plasma with less than 0·15 u. of kallikrein, using the isolated guinea-pig ileum as a test object. This was observed regularly either when plasma (0·2–0·5 ml.) was incubated with kallikrein for 1 min and then tested, or when 0·5 ml. plasma was added to the bath containing the ileum and the kallikrein then added to the bath contents. In this case the final concentration of kallikrein was approximately 0·01 kallikrein u./ml. or 0·3 μg protein/ml. of our purer preparation. In terms of the purest preparations described by the German workers (Frey *et al.* 1950), this represents a concentration of less than 0·01 μg kallikrein/ml. The release of kallidin by serum kallikrein is readily prevented by incubation of the latter with SBTI. Text-figure 2 shows the kallidin-releasing action of kallikrein on heated guinea-pig plasma and the inhibition of this release by preliminary incubation of the kallikrein solution wilh SBTI. This figure also shows that after inhibition of release by SBTI, kallidin may still be released from a mixture of plasma and inactivated kallikrein by addition of excess kallikrein.

A comparison of a potent fibrinolytic preparation of human plasmin (see below) with serum kallikrein indicated that the latter was at least several hundred times the more active of the two in releasing kallidin from heated (56° C, 3 hr) guinea-pig or human plasma. In fact, even when 1 mg plasmin was added to the test bath containing heated plasma, evidence of

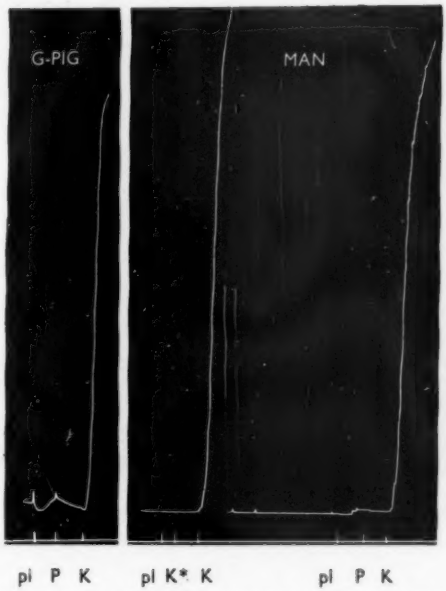
release of a smooth-muscle stimulant was doubtful. Similar results were obtained with unheated plasma. The rapid release of kallidin from guinea-pig and from human plasma under conditions when equal or greater amounts of plasmin were ineffective is shown in Text-fig. 3. This figure

Text-fig. 2. The release of kallidin by serum kallikrein and inhibition of release by soya-bean trypsin inhibitor (SI). Contractions of guinea-pig ileum in 17 ml. bath of Mg-free Tyrode solution (34° C) containing atropine and mepyramine (10^{-5} g/l.). Continuous line indicates bath not washed out. pl, 0·5 ml. dialysed, heated (56° C, 3 hr) guinea-pig plasma; K, serum kallikrein (0·5 u. 200 μg); K + SI, serum kallikrein (0·5 u.) incubated with soya-bean trypsin inhibitor (200 μg/ml., (*a*); 20 μg/ml., (*b*)) for 10 min at 35° C before addition to bath.

also shows that kallikrein, if the pH of the solution is first lowered with hydrochloric acid to pH 2 for 20 min, is completely inactivated. Plasmin, however, is reported to be very stable at this pH (Müllertz, 1956). Human salivary kallikrein and crystalline trypsin preparations were also very effective in releasing a smooth-muscle stimulant from heated guinea-pig or human plasma under these conditions.

Fibrinolytic activity

The fibrinolytic activity of plasmin, trypsin, serum kallikrein and salivary kallikrein was measured on fibrin plates. Plasmin was the most effective of these on a weight basis, regularly producing detectable lysis when 0·03 ml. of a solution containing 0·75 μg/ml. was placed on a fibrin plate. Such a solution of plasmin produced a lysed ring of fibrin with a mean diameter of 6·6 mm. With 0·03 ml. volumes, containing plasmin in concentration of 10

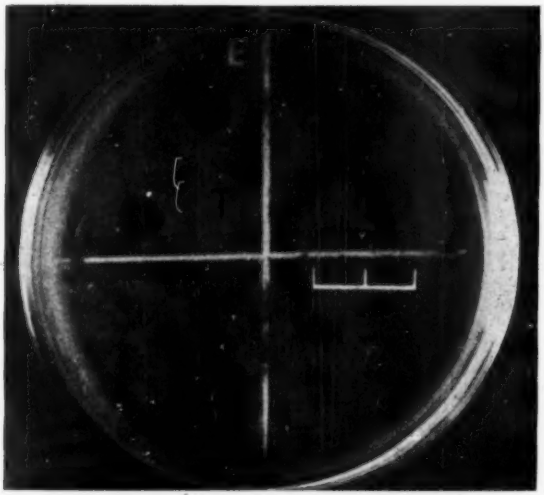


Text-fig. 3. Contractions of isolated guinea-pig ileum in Tyrode solution containing atropine and mepyramine. Continuous line indicates bath not washed out. The release of kallidin from guinea-pig (left) and human plasma (right). pl, dialysed heated plasma (0·5 ml. g-pig; 1·0 ml. man) P, human plasmin (1·0 mg); K, serum kallikrein (0·25 u.); K*, serum kallikrein (0·25 u. + HCl) at pH 2 for 20 min before neutralization and addition to bath.

and 20 μg/ml., the mean diameters of the lysed areas were approximately 10·9 and 15·0 mm, respectively. Trypsin was also detectable in solutions containing 0·75 μg/ml., but the lysed area was smaller than with plasmin. The minimal concentration of trypsin detectable was approximately five times greater than that of plasmin. Serum kallikrein showed fibrinolytic activity in high concentrations, but activity was not demonstrated until concentrations of 15–50 μg/ml. were employed. The activity at this concentration was less than that of solutions containing 0·75 μg/ml. trypsin

or plasmin. The relative activities of plasmin, trypsin, and kallikrein in lysing human fibrin are shown in Text-fig. 4.

The fibrinolytic activity of trypsin and plasmin were both sensitive to SBTI, effective concentrations of each of these enzymes being completely inactivated by the same or even lower concentrations of SBTI. Both enzymes showed a much less sensitivity to OI, although the fibrinolytic activity of trypsin was reduced by high concentrations of this inhibitor.



Text-fig. 4. Lysis of human fibrin (16 hr, 37° C); 0·03 ml. of each solution in concentration of 10 μg/ml. P, human plasmin; T, crystalline trypsin; K, serum kallikrein.

DISCUSSION

Our highly purified preparations of bradykinin, though not yet pure, were more effective than histamine on a weight basis in increasing capillary permeability in the lower concentrations. It is difficult, however, to compare the relative potency of histamine and of bradykinin in increasing permeability because of the different shapes of the dose–response curves, and because of the apparent greater accumulation of dye in the lesions produced by bradykinin. Since pure bradykinin has been estimated to contain 5000–10,000 u./mg, it would be 5–10 times still more potent than our preparation. Bradykinin preparations, which varied from 7 to 1000 u./mg (assayed on the guinea-pig ileum) showed parallel gut-stimulating and permeability-enhancing properties, indicating that these properties reside in the same molecule. The recently prepared pure bradykinin has, in fact,

been found to retain the permeability-increasing activity (D. F. Elliott, E. W. Horton & G. P. Lewis, personal communication).

The remarkable activity of serum kallikrein in increasing permeability is of interest in so far as high concentrations of this substance exist in blood in an inactive but activable form (Frey *et al.* 1950). The close similarity of its dose–response curve to that of bradykinin (Text-fig. 1) suggests that this effect is due to the release of the polypeptide, kallidin, which kallikrein releases from a precursor in plasma and lymph (see Werle, 1960; Schachter, 1960). This view is supported by the fact that our most active serum kallikrein preparation was more active in releasing kallidin than trypsin was in releasing bradykinin, and that this property, like the permeability-increasing property, was greatly reduced by incubation with SBTI but not with OI.

The fact that serum kallikrein is probably an enzyme which is present in an inactive but activable form in plasma raises the question of its possible relationship to plasmin, the fibrinolytic enzyme (or enzymes) in plasma (Müllertz, 1956; Biggs & MacFarlane, 1957). This is particularly relevant since it has been stated that human plasmin is a very potent releaser of plasma kinin (Lewis, 1958) although this release appears to be rather slow. Our experiments indicate that there is no correlation between fibrinolytic activity and the rapid release of a smooth-muscle stimulant from plasma by serum kallikrein, plasmin, trypsin and salivary kallikrein. In fact, plasmin, which had the greatest fibrinolytic activity, was ineffective in releasing a smooth-muscle stimulant from plasma in concentrations approximately 500 times greater than that in which our best serum kallikrein preparation released kallidin within a few seconds of its addition to plasma. These facts indicate that serum kallikrein has different substrate requirements from that of the highly active fibrinolytic enzyme of plasma. It is of interest in this connexion that human salivary kallikrein, a potent releaser of kallidin, completely failed to lyse human fibrin, even in high concentrations. Further chemical studies are desirable to clarify the interesting problem of the substrate specificities of the different kallikreins.

The prominent pharmacological properties of serum kallikrein (and of the other kallikreins in so far as they have been studied) are the vasodilator, permeability-enhancing, and kallidin-releasing actions. It seems unlikely that these properties will dissociate with purification (Habermann, 1959). The suggestion (Schachter, 1956; Herxheimer & Schachter, 1959) that the release of kallidin-like substances from plasma by dilution, or by contact with glass, is due to the activity of kallikrein, is strengthened by our demonstration of the potent kallidin-releasing action of serum kallikrein. Similarly, the permeability-enhancing activity of diluted plasma (Mackay, Miles, Schachter & Wilhelm, 1953; Miles & Wilhelm,

1955) and of the permeability globulins in fractionated plasma (Wilhelm, Miles & Mackay, 1955; Mill, Elder, Miles & Wilhelm, 1958), or in inflammatory exudates (Spector, 1957), may all be due to serum kallikrein. For the same reasons it is possible that the 'enzyme' activated in plasma by glass contact (Armstrong, Jepson, Keele & Stewart, 1957), which causes the release of plasma kinin and accelerates clot formation (Margolis, 1958), is also serum kallikrein. Also, those individuals whose blood shows a prolonged clotting time *in vitro*, and who show no haemorrhagic disturbance, but are deficient in the so-called Hageman factor in their plasma (Ratnoff & Colopy, 1955) may, in fact, be deficient in the inactive kallikrein precursor, since their plasmas do not release plasma kinin on contact with glass or kaolin (Margolis, 1958). It would be of interest, therefore, to measure the amount of active kallikrein extractable from their blood by the usual procedures (Frey *et al.* 1950). If serum kallikrein is in fact the active agent involved in all these observations, a possibility which seems not unlikely, it would provide a simplified explanation of many diverse and apparently unrelated observations. More experiments are required to establish definitely whether or not this is the correct explanation of these facts.

SUMMARY

1. Bradykinin, when injected intradermally, is one of the most effective enhancers of capillary permeability, as measured by its ability to increase permeability to a circulating dye in the guinea-pig. The effect differs from that of histamine in that there is a greater accumulation of dye in the lesions, and that there is an earlier pronounced flattening of the dose-response curve in respect to lesion size. Its action is unaffected by treatment of the animal with mepyramine or by incubation of the bradykinin solution with soya bean or ovomucoid trypsin inhibitors.

2. Serum kallikrein is also very effective in increasing capillary permeability in the guinea-pig. Its effects resemble those of bradykinin both in the appearance of the lesion and in the shape of the dose–response curve. This action of kallikrein is unaffected by treatment of the animal with mepyramine but is reduced if the solutions are incubated with soya-bean trypsin inhibitor before injection. Ovomucoid inhibitor is without effect. These results suggest that this action of kallikrein is mediated by the release of kallidin from plasma. Serum kallikrein is a very potent releaser of kallidin from heated (56° C, 3 hr) or unheated plasma or serum. This action is readily suppressed by incubation with soya-bean trypsin inhibitor but not by ovomucoid inhibitor.

3. Plasmin is practically without effect on capillary permeability, and fails to release a smooth-muscle stimulant from plasma under conditions

in which trypsin, salivary kallikrein and serum kallikrein are very effective. There is no relationship between the ability of these substances to produce lysis of fibrin and to release kallidin or bradykinin.

4. The possible activation of serum kallikrein in a number of procedures (glass contact, dilution, etc.) which result in the appearance of smooth-muscle-stimulating or permeability-enhancing activity in plasma is discussed. Also, the possible relationship of various other substances in plasma (permeability globulins, contact factor, Hageman factor) to serum kallikrein is considered.

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EXPLANATION OF PLATE

Fig. 1. Skin of guinea-pig with circulating pontamine blue dye showing lesions produced by intradermal injections (0·1 ml.) of B, bradykinin; H, histamine; K, serum kallikrein.

Fig. 2. Skin of guinea-pig with circulating pontamine blue dye. Lesions produced by intradermal injections (0·1 ml.) of H, histamine; P, human plasmin.

Fig. 3. Skin of both flanks of same guinea-pig with circulating pontamine blue dye. Right: skin of one flank with intradermal injections of approximately equal activities of bradykinin (B), histamine (H), and serum kallikrein (K). Left: skin of other flank similarly injected after mepyramine (0·1 mg/kg) had been administered intravenously; only the histamine response is suppressed.

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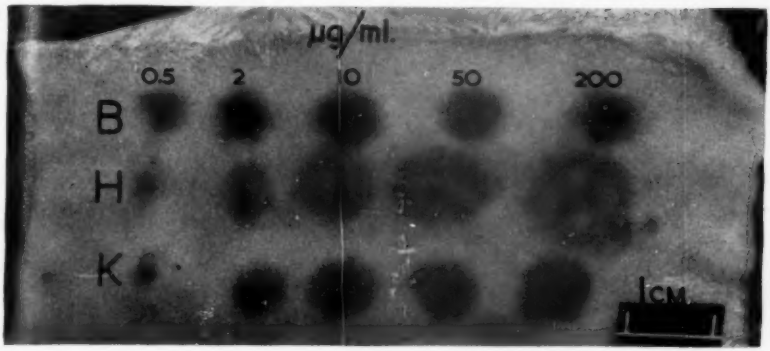

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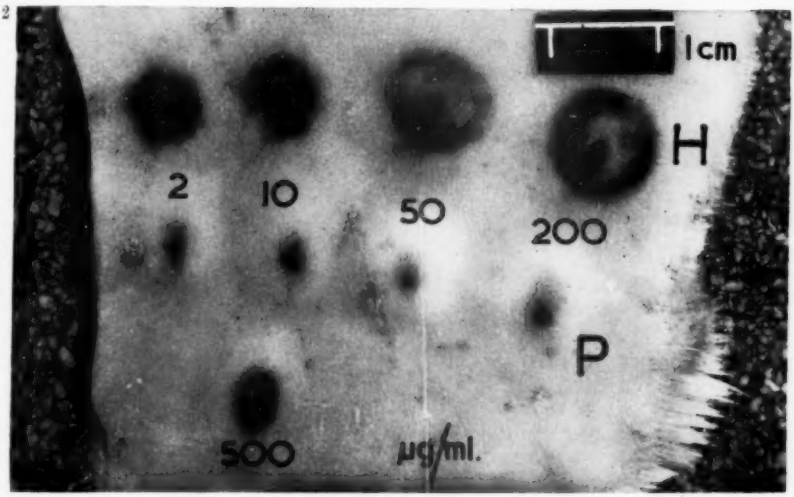

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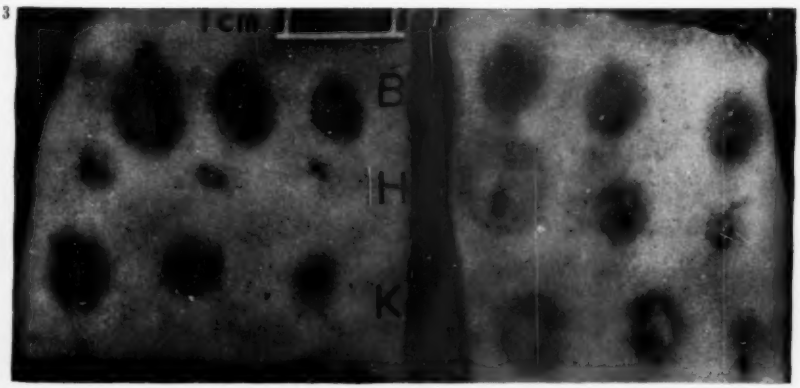

K. D. BHOOLA, J. D. CALLE AND M. SCHACHTER (*Facing p.* 86)

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CARDIOVASCULAR AND RESPIRATORY RESPONSES TO HEATING THE CAROTID BLOOD

BY P. P. NEWMAN AND J. H. WOLSTENCROFT

From the Department of Physiology, School of Medicine, University of Leeds

(*Received* 9 *December* 1959)

Changes in breathing produced by heating the carotid blood were first reported by Goldstein (cited by Fick, 1872) and Fick (1872) after Ackermann (1867) had investigated respiratory effects produced by heating the whole animal; changes in blood pressure and heart rate were later described by Kahn (1904) and Moorhouse (1911). A fall of blood pressure when the whole animal was heated was briefly reported by Heymans (1921) and Rodbard (1948).

The location of regions responding to temperature changes has been the subject of many investigations. Since the work of Barbour (1912) attention has been directed towards the hypothalamus, heating of which has been shown to produce vascular and respiratory effects (Magoun, Harrison, Brobeck & Ranson, 1938; Ström, 1950). In the present work a fall of blood pressure and increased respiration have been produced by heating the carotid blood in anaesthetized cats, and in decerebrate cats in which the hypothalamus had been removed. The evidence to be presented suggests that these effects were mediated by the medulla oblongata. Some preliminary results have already been reported (Newman & Wolstencroft, 1956, 1957).

METHODS

Experiments were carried out on forty-eight cats of either sex weighing between 1·5 and 5 kg and anaesthetized with sodium pentobarbitone I.P. (Nembutal; Abbott Laboratories, 45 mg/kg body weight). Some animals were decerebrated at the level of the superior colliculi, with the carotid arteries temporarily clamped, care being taken to ensure removal of the hypothalamus. In some animals the spinal cord was divided immediately below the medulla. The femoral arterial blood pressure was recorded with either a mercury manometer, a Hürthle membrane manometer or a capacitance manometer coupled to a pen-writer. Heart rate was recorded with an ink-writing electrocardiograph. The trachea was cannulated and the animal breathed through valves. Respiration was recorded by a tambour attached to a side arm of the outlet tube from the expiratory valve. The presence or absence of sweating was tested (*a*) by dusting the foot pads with Quinizarin compound (Burroughs Wellcome) which turns from grey to violet in the presence of water, or (*b*) by measurement of skin resistance from foot pad to leg.

Carotid heating. The method of heating the carotid blood was as follows: one or both common carotid arteries were cannulated proximally and the blood passed through a spiral glass tube in a water-bath before being returned through a cannula inserted into the distal end of the same artery. Heating was effected by gradually raising the temperature of the water-bath surrounding the spiral from 38 to 55° C; cooling was effected by replacing with cold water. This method avoids the danger of spread of heat to the vagi and other structures in the neck, which occurs when heating tubes are placed around the carotid arteries (Sihler, 1879).

Temperature measurement. Thermocouples were made by brazing together 40 s.w.g. copper and Constantan wires. One thermocouple was inserted deep into the substance of a cerebral hemisphere of the intact cat or into the upper brain stem of the decerebrate cat. A second thermocouple was inserted through the central end of a lingual artery so that its tip lay in the external carotid artery. Rectal temperature was measured with a mercury thermometer.

RESULTS

Heating the blood passing through the carotid arteries produced circulatory and respiratory changes.

Cardiovascular effects

When heating commenced the arterial blood pressure remained constant until the temperature recorded by the thermocouple in the brain was between 41 and 42° C. The temperature recorded in the lingual artery was usually 1° C higher. At this point an abrupt fall in blood pressure of 40–70 mm Hg occurred (Fig. 1). If the increased brain temperature was maintained the blood pressure remained depressed, but when heating ceased and the blood and brain cooled to their original temperatures the blood pressure returned to its previous height. This response to heating and cooling could be obtained repeatedly in the same animal. The rectal temperature did not rise more than 0·2° C.

The same effect on the arterial blood pressure was obtained in the decerebrate animal (Fig. 2); but not after section of the cervical cord, even in animals with a blood pressure over 100 mm Hg.

In the intact animal the response to heating occurred after bilateral section of the carotid sinus nerves, the glossopharyngeal and hypoglossal nerves. The response was also present after section of both cervical vago-sympathetic nerves. The heart rate either remained unchanged during the period of heating or was reduced, e.g. from 210 to 180 beats/min, during the fall in blood pressure. It was never increased.

Respiratory effects

As the temperature of the blood in the carotid artery began to rise, an increase in the depth of respiration was usually observed with little or no change in rate. The increase in depth became marked when the blood pressure fell and it was then sometimes accompanied by an increase in

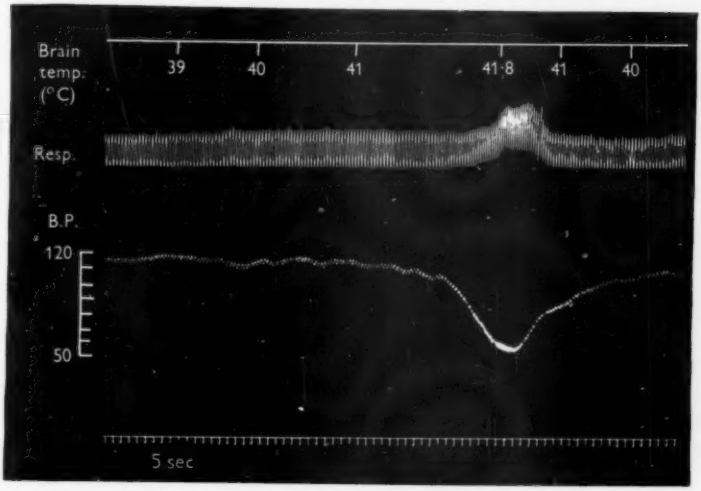

Fig. 1. The effect of heating the carotid blood on respiration and blood pressure in a cat under Nembutal anaesthesia. As the brain temperature began to rise an increase in the depth of respiration occurred, the increase in depth being marked when the blood pressure fell. A fall in blood pressure occurred when the brain temperature reached 41·5° C. Respiration and blood pressure returned to original levels on cooling. From above down: brain temperature; respiration; blood pressure (mm Hg); time marker, 5 sec.

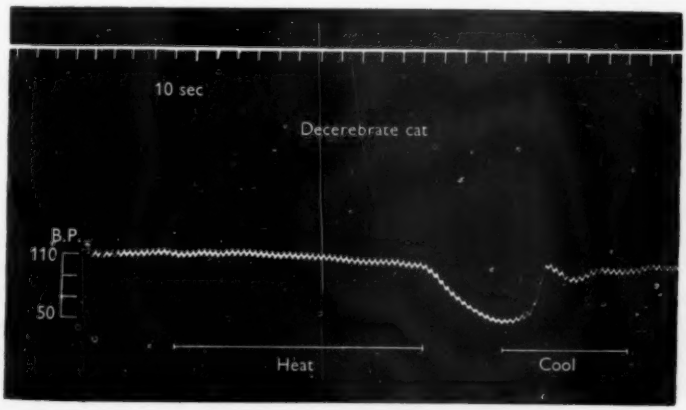

Fig. 2. Decerebrate cat. The effect on femoral blood pressure of heating and cooling the carotid blood. B.P. (mm Hg); time marker, 10 sec.

rate (Fig. 1). These effects were not abolished by section of the carotid sinus nerves. To test whether the fall in blood pressure was secondary to loss of CO_2 due to the increased depth of respiration, constant lung ventilation was maintained by a pump during heating. This procedure did not affect the fall in blood pressure.

Sweating

During heating of the carotid blood there was no obvious sign of sweat on the foot pads and none could be detected by the use of Quinizarin compound or by measurements of skin resistance.

DISCUSSION

It is rather surprising that, among the various reports on the effect of heating the blood passing to the head, none include a description of the sudden fall in blood pressure described above. The only comparable effects appear to be those reported by Heymans (1921) and Rodbard (1948). Heymans (1921) heated the whole body, by surrounding an artificial carotid-jugular anastomosis with hot water, in rabbits, dogs and cats. The hyperthermia so produced was fatal when the rectal temperature exceeded 42·5–42·7° C and a sudden fall in blood pressure occurred at this point. Rodbard (1948) reported that hyperthermia was fatal at about 3° C above body temperature in chickens, dogs and rabbits; he attributed death to a fall of blood pressure.

Relatively little attention has been given previously to the possible site of action of the heated blood. The hypothalamus was not involved in the fall in blood pressure, since it occurred in the decerebrated animals. The effect was not due to a reflex from the carotid sinus or carotid body, since it was present after bilateral section of the lower four cranial nerves. It has recently been found that local heating of the medulla can produce a similar fall in blood pressure (Holmes, Newman & Wolstencroft, 1960). Finally, the fall in blood pressure produced by heating the carotid blood was abolished by section of the spinal cord. Thus it seems probable that the heated blood produces its effect by action on the medulla. The fact that blood flowing in the carotid arteries of intact and decerebrate cats may reach the medulla, particularly on heating, has been demonstrated by Holmes, Newman & Wolstencroft (1958).

The increase in breathing could be due to a direct central action of increased temperature. However, it could also be explained by changes in CO_2 tension and acidity produced by heating the blood on its way to the head. Calculations with the data of Rosenthal (1948) and of Brewin, Gould, Nashat & Neil (1955) show that a rise of 3° C in the temperature

of the carotid blood would be likely to decrease its pH by 0·04 and increase its CO_2 tension by about 7 mm Hg. Both of these changes would stimulate respiration. Another possible factor is that an increase in respiration may be secondary to a fall in blood pressure. This could provide an explanation of the sudden increase in rate reported in anaesthetized dogs at 41·7° C rectal temperature (Richet, 1898) and in anaesthetized calves at 40·5° C rectal temperature (Bligh, 1957).

It may be significant that the fall in arterial blood pressure was never associated with an increase in heart rate, and that heart rate was often reduced. This suggests that the response may be due to a simultaneous action on cardiac and vasomotor mechanisms. It is interesting to compare the effects produced in animals with similar effects resulting from the action of heat in human subjects. Hill & Flack (1909) recorded a fall in systolic pressure produced by immersion of subjects in hot baths. Bazett (1927) reviewed other reports of changes in blood pressure in response to heating. The present results may have some relevance to the syndrome of heat exhaustion in man, as distinct from that of heat stroke (Ferris, Blankenhorn, Robinson & Cullen, 1938). A frequent occurrence in heat exhaustion is a low systolic blood pressure, often associated with syncope on standing (Ladell, Waterlow & Hudson, 1944; Borden, Waddill & Grier, 1945). The condition is, of course, more complicated than that described in the present series of experiments, since heating may produce general vasodilatation together with a reduction in blood volume due to dehydration. However, an action of the heated blood similar to that observed in animals is one possible cause of the fall in blood pressure that occurs in heat exhaustion.

SUMMARY

1. The blood flowing in the carotid arteries of anaesthetized cats was heated. If the temperature in the brain rose to 41–42° C there was a sudden fall in blood pressure of 40–70 mm Hg. Heart rate was unchanged or slightly reduced. Respiration was increased. Sweating could not be detected.

2. The fall in blood pressure was present in the decerebrate cat with hypothalamus removed, but not in the spinal cat. It was present after bilateral section of the carotid sinus nerves and of the lower four cranial nerves.

3. It is possible that the heated blood produces its effects by acting on the medulla.

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A HEAT-SENSITIVE REGION IN THE MEDULLA

BY R. L. HOLMES, P. P. NEWMAN AND J. H. WOLSTENCROFT
From the Departments of Anatomy and Physiology, School of Medicine, University of Leeds

(*Received* 9 *December* 1959)

A previous paper (Newman & Wolstencroft, 1960) described a fall in arterial blood pressure and increased respiration which were produced by heating the carotid blood in the anaesthetized cat. Since this effect was present in the decerebrate but not in the spinal animal, the effect of localized heating of the lower brain stem was then investigated. A region was found where heating produced changes in blood pressure and respiration similar to those produced by heating the carotid blood. These experiments are described in this paper; preliminary reports have been given by Newman & Wolstencroft (1957) and Holmes, Newman & Wolstencroft (1958).

METHODS

Experiments were performed on twenty-eight cats of either sex weighing between 2 and 4·5 kg and anaesthetized with sodium pentobarbital (45 mg/kg). Six cats were decerebrated at the level of the superior colliculi. Blood pressure was recorded from the femoral artery by means of a mercury manometer. Respiration was recorded by a method previously described (Newman & Wolstencroft, 1960).

The pons and medulla were exposed by a ventral approach through the soft structures of the neck followed by removal of the overlying bone. Localized heating was effected by an electrode held in a stereotaxic instrument and connected to the output of a high-frequency (1500 kc/s) oscillator. The heating electrode consisted of silver wire, 0·2 mm diameter, insulated except at the tip. The other electrode was a saline pad applied to one limb. The temperature 2–3 mm from the electrode tip was recorded by a thermocouple inserted into the brain by means of forceps. Thermocouples were made by brazing together 40 s.w.g. copper and Constantan wires. Owing to electrostatic pick-up the thermocouple may have given readings which were 1° C higher than the true temperature (Huntoon, 1937).

Electrode placements were determined by histological study after fixing the brain stem in formalin and embedding in celloidin or paraffin wax. Serial sections were cut at 25–100 μ and stained with toluidin blue or thionine. The track of the electrode could easily be recognized in these sections.

RESULTS

Effect of heating

The temperature recorded by the thermocouple before heating began was usually about 38° C. At responsive points in the medulla the following effects were observed when the heating current was turned on: Respiration increased with the rise in temperature, but the arterial blood pressure

showed no significant change until the temperature recorded by the thermocouple reached about 41° C. At this temperature the blood pressure fell about 40–60 mm Hg (Fig. 1). Heating was then discontinued and the blood pressure and respiration returned to their original state. During the fall in blood pressure bradycardia was sometimes observed. It was possible to repeat this response to heating several times with the electrode in the same position. The fall in blood pressure could be obtained when lung ventilation was kept constant by means of a pump. Rectal temperature remained unaltered during the period of heating.

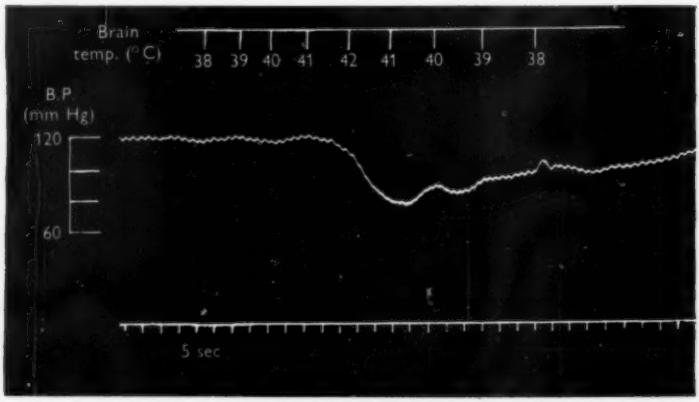

Fig. 1. Direct heating of the medulla. A fall in blood pressure of 40 mm Hg occurred when the temperature recorded by the thermocouple reached 41·5° C. The blood pressure returned to its original level as heating was discontinued. From above down: brain temperature; femoral blood pressure; time marker, 5 sec.

Location of responsive regions

To determine the distribution of responsive points the brain stem was explored within the limits of the exposed field which extended from the upper pons to the lower medulla. In a limited region of the upper medulla close to the mid line falls of blood pressure of 40–60 mm Hg were obtained on heating to 41–42° C (Fig. 2). Smaller responses occurred in the lower pons and the most rostral part and middle third of the medulla. In these regions respiration was increased but the fall in blood pressure was not more than 20 mm Hg at 41–42° C and could not be reproduced consistently, although heating to 43° C occasionally produced larger responses. The latter effect could have been due to the spread of heat, since it was found that a thermocouple placed 1 cm from the heating electrode showed a temperature which was about 1° C lower than that recorded close to the electrode. Heating in the upper pons and caudal medulla did not produce an increase in respiration or a fall in blood pressure (Fig. 2).

In six experiments on decerebrate cats localized heating was performed after sections at various levels. Section of the brain stem at the junction between pons and medulla abolished the response to heat applied to points above but did not abolish the response to heat from points below the section. Similar results were obtained after sections through the upper medulla. The lower limit of the responsive region could not be demonstrated in this way as the sections produced cessation of respiration and variable changes in blood pressure.

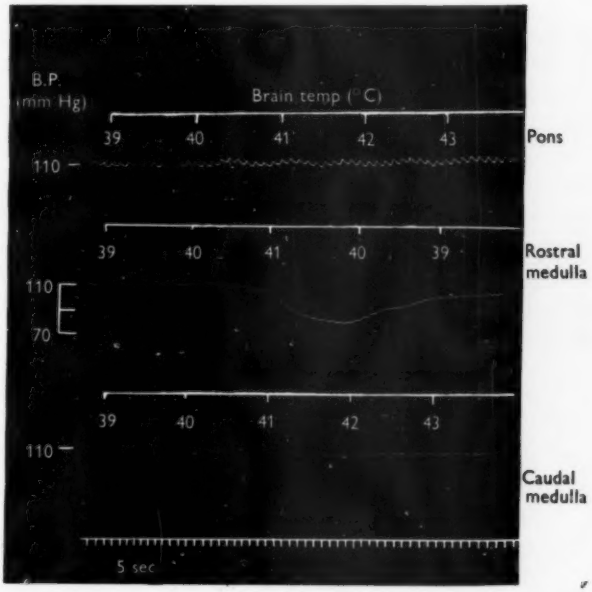

Fig. 2. Direct heating of the brain stem to determine the distribution of responsive points. With the heating electrode in the upper pons or in the caudal medulla, the blood pressure remained unchanged. The brain temperature recorded was 43° C. With the heating electrode in the rostral medulla a fall of blood pressure occurred at 41° C.

Histological studies. Examination of serial sections through the brain stem showed that in each instance in which heating had produced a fall in blood pressure the electrode had entered the medial reticular formation at some point rostral to the hypoglossal nucleus. In those experiments in which the largest responses had been obtained the electrode had passed through the fibres of the trapezoid body, or near to its lower border, so that the tip lay surrounded by cells of the nucleus reticularis gigantocellularis (Fig. 3*B*), in a plane between the rostral part of the nucleus ambiguus and nucleus of the VI nerve. Smaller responses had followed

insertions of the electrode rostral to the plane of the nucleus of the VI nerve (Fig. 3*A*), where its tip had entered the nucleus reticularis pontis caudalis, or the nucleus reticularis tegmenti pontis. Similar responses had also been obtained in experiments where the electrode was more caudally placed, in relation to the lowest cells of the nucleus reticularis gigantocellularis; insertions in the lower part of the medulla, below the level of this latter nucleus, failed to elicit reversible responses.

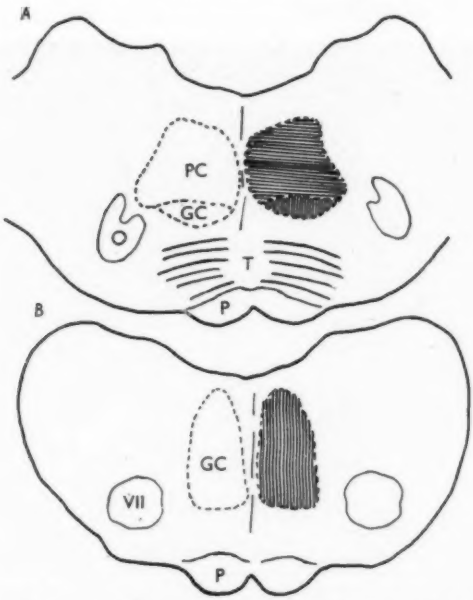

Fig. 3. Diagrams of transverse sections through the brain stem of the cat; *A* at the level of the rostral part of the superior olive, *B* at the level of the motor nucleus of VII cranial nerve. Shaded areas represent parts of the medial reticular formation. PC, nucleus reticularis pontis caudalis; GC, nucleus reticularis gigantocellularis; O, superior olive; T, trapezoid body; P, pyramid; VII, motor nucleus of facial nerve.

DISCUSSION

The experiments demonstrate that structures sensitive to increased temperature are present in the brain-stem reticular formation. Although the reticular formation was long regarded as a diffuse system of cells and fibres it is now apparent that considerable intrinsic organization exists (see Brodal, 1957). The experiments reported here suggest that a group of nerve cells distributed throughout the nucleus reticularis gigantocellularis bring about a vasodepressor response when stimulated by the local application of heat. Points heated in the caudal pons and middle medulla

gave smaller responses. This may be due either to the spread of heat to the primary responsive region or may indicate fewer responsive cells in the regions adjacent to it. Histologically there is no clear demarcation between the nucleus reticularis pontis caudalis and the nucleus reticularis gigantocellularis. In the cat, descending (reticulospinal) fibres arise from the entire nucleus reticularis gigantocellularis and also from the nucleus reticularis pontis caudalis (Torvik & Brodal, 1957). The descending medullospinal fibres might provide a pathway for the mediation of the depressor response here described.

The anatomical site of the 'heat-sensitive' region corresponds with 'depressor' or 'vasodilator' points located by several investigators. Wang & Ranson (1939) recorded blood-pressure changes in the cat following electrical stimulation of the brain stem. They found pressor points in the lateral portion and depressor points in the medial portion of the reticular formation; however, a predominantly 'depressor' region sometimes produced a rise in blood pressure. Alexander (1946) identified in the cat depressor points near the mid line of the rostral medulla. Lindgren (1955) showed in the cat that brain stem stimulation can activate a vasodilator pathway to the skeletal muscles. Thus there is good evidence that electrical stimulation of discrete points in the brain-stem reticular formation can produce vasodilation and a fall of blood pressure.

In the present experiments it was found that points giving reversible responses to heat were restricted to a limited region of the medulla and the effect was always a fall, and never a rise, of blood pressure. Nothing is known of the neuronal mechanisms involved. The heat-sensitive elements may induce inhibition of the vasoconstrictor neurones or may actively excite the vasodilator systems described by Lindgren (1955) and others.

It is interesting that respiratory stimulation was maximal when the heating electrode was in the reticular formation of the medulla rostral to the region of the 'inspiratory centre' (Pitts, Magoun & Ranson, 1939). A direct action of heat on structures in this part of the lower brain stem is a possible explanation of the increase in respiration that occurs with rise of temperature in the decerebrate animal. However, the present experiments do not provide unequivocal evidence on this point, since the respiratory stimulation could have been due, in part or in whole, to a rise in local CO_2 tension brought about by the local rise in temperature.

In the classical experiments of Magoun, Harrison, Brobeck & Ranson (1938) activation of heat-loss mechanisms by local heating of the hypothalamus was described. Negative results were obtained on local heating in other regions as far caudal as the upper pons; but the lower pons and the medulla were not explored. The present study does not provide evidence of brain-stem influences on heat regulation, although it is interesting to

note that Keller (1938) reported the existence of a heat-loss mechanism in the reticular formation of the chronic mid-brain cat with hypothalamus removed. It would, however, be premature to deduce that heat-sensitive structures in the medullary reticular formation have any regulatory function.

SUMMARY

1. In cats under sodium pentobarbital anaesthesia the pons and medulla were explored with a heating electrode, and circulatory and respiratory responses were observed.

2. On heating certain regions respiration was increased and a fall in blood pressure of 40–60 mm Hg occurred when the temperature recorded by a thermocouple reached 41–42° C. When heating was discontinued the blood pressure and respiration returned to their original state.

3. The region of maximal response was in the upper and medial reticular formation of the medulla at the level of the trapezoid body. Histologically the region was identified as the nucleus reticularis gigantocellularis.

4. It is suggested that structural elements which are sensitive to heating are situated in the medullary reticular formation.

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EFFECT OF DENERVATION ON RESPIRATORY ENZYMES IN SALIVARY GLANDS

BY I. NORDENFELT, P. OHLIN AND B. C. R. STRÖMBLAD
From the Institute of Physiology, University of Lund, Sweden

(*Received* 14 *December* 1959)

Section of the efferent nerves has been found to cause changes in the activity of cholinesterase and amine oxidase in various organs (Strömblad, 1955*b*, 1956). Changes in the activity of these two enzymes, which are of importance in the metabolism of transmitter substances, could be specifically caused by denervation or could be part of general changes of enzymic activity after denervation. For this reason the effect of nerve section on the activity of other enzymes present in salivary glands of the cat and rabbit has been studied and the results compared with earlier observations on amine oxidase and cholinesterase activity in the same tissues. The enzymes selected were succinic dehydrogenase, cytochrome oxidase and fumarase, which are all important in the respiratory activity of mammalian cells.

METHODS

Cats weighing 2·5–4 kg and rabbits weighing 2·0–3·5 kg were used. The parotid gland of the cat and the submaxillary glands of both cat and rabbit were studied. To obtain sympathetically denervated glands the right superior cervical ganglion was excised. Parasympathetic denervation of the submaxillary glands was achieved by division of the right chorda tympani (preganglionic pathway). The parotid gland of the cat was parasympathetically denervated either preganglionically by destruction of the right tympanic plexus or postganglionically on the left side by section of the auriculotemporal nerves. The operations were done with aseptic precautions under ether or pentobarbitone (Nembutal; Abbott Laboratories, Ltd.) anaesthesia. The operations on the parotid gland of the cat were carried out as described by Strömblad (1955*a*). Three to five weeks after the operation the enzyme activities were estimated. The cats were anaesthetized with chloralose after administering ether. The rabbits were killed by a blow on the neck. The glands were removed, cleaned, washed in saline solution, dried between filter papers and weighed. They were immediately minced with scissors and homogenized in glass homogenizers with 2–3 volumes of distilled water. The volume was finally adjusted to 10 ml. with distilled water.

Estimations of enzymic activity

Succinic dehydrogenase. This was estimated manometrically by the Warburg technique in reaction vessels of conventional size. The main chamber contained the extract (0·3 ml. cat-gland or 0·5 ml. rabbit-gland homogenate), 0·4 ml. 10^{-4} M cytochrome C, 0·02 ml. 3·13 % $MgCl_2$ and 0·067 M sodium phosphate buffer of pH 7·4 to bring the volume to 2·7 ml. The side arm contained 0·3 ml. 0·5 M sodium succinate of pH 7·4 and the centre well 0·3 ml.

M-KOH. The gas phase was O_2 and the temperature 37° C. Readings were taken every 5 min for 30 min and were corrected for changes in the thermobarometer and in the enzyme blank. All estimations were made in duplicate. The activity was expressed in units per gland or per gram of tissue. One unit is defined as the amount of enzyme which utilizes 1 μl. O_2 in 30 min at 37° C.

Cytochrome oxidase. This was also determined manometrically. The side arm contained 0·3 ml. 4×10^{-4} M cytochrome C and the centre well 0·3 ml. M-KOH. Besides homogenate the vessels contained in the main compartment 0·3 ml. 4×10^{-3} M-$AlCl_3$, 0·3 ml. 0·014 M sodium ascorbate (pH 7·0) and 0·067 M sodium phosphate buffer (pH 7·4). The homogenate was diluted 10 times and 0·2 ml. was used in two flasks, 0·8 ml. in another pair of flasks, and the volume adjusted with buffer to give final reaction volumes of 3·0 ml. Readings were taken every 5 min for 30 min and the usual corrections applied. The enzymic activity was calculated from the difference in O_2 consumption in the flasks containing different amounts of tissue (Umbreit, Burris & Stauffer, 1957). The unit of activity is defined as for succinic dehydrogenase.

Fumarase. This enzyme was determined spectrophotometrically, as described by Racker (1950). The original homogenate was diluted 20 times with distilled water and centrifuged at 900 *g* for 1 hr. One ml. of the supernatant, 1 ml. of 0·067 M sodium phosphate buffer of pH 7·4 and 1 ml. of 0·15 M sodium L-malate were mixed in the cuvette, and the changes in absorption at 240 mμ were measured in a Beckman DU spectrophotometer. Readings were taken every 15 sec for 3 min. One unit of activity is defined as the amount of enzyme which produces a change in optical density at 240 mμ of 0·001/min.

RESULTS

Normal glands

There were marked differences in enzymic activities between the three types of glands used. Thus, when expressed on a weight basis, much the highest succinic dehydrogenase activity was found in the submaxillary gland of the cat, whereas the highest cytochrome oxidase and fumarase activities were in the submaxillary gland of the rabbit. The results are given in Table 1.

Even in the same species large differences in enzyme activities were found when the glands of one animal were compared with the corresponding glands of another. However, the two submaxillary glands or the two parotid glands of the same animal showed much the same activities. For this reason the effect of denervation was studied on such pairs of glands; one gland was denervated and the contralateral served as a control. No significant sex differences were found.

Parasympathetic denervation

Cat submaxillary gland. Three to five weeks after section of the chorda-lingual nerve all three enzyme activities, when expressed on a whole-gland basis, were decreased considerably in the denervated glands (Fig. 1). It is well known that the weight of the submaxillary gland is reduced after section of the chorda. In the six cats of this series the mean weight of

the denervated glands was 60% of the normal. The decreases in total activities, however, were much greater than this. Consequently, the enzyme activities (on a gram basis) were also reduced, although the fall in fumarase activity was not significant.

TABLE 1

Gland	Succinic dehydrogenase activity (u.)		Cytochrome oxidase activity (u.)		Fumarase activity (u.)		Fresh weight of gland (g)
	Per gland	Per gram fresh wt.	Per gland	Per gram fresh wt.	Per gland	Per gram fresh wt.	
Cat submaxillary	3826 ± 347 (n = 22)	3023 ± 243	7344 ± 538 (n = 21)	6002 ± 539	5220 ± 361 (n = 22)	4264 ± 233	1·22 ± 0·04 (n = 28)
Cat parotid	1000 ± 60 (n = 33)	990 ± 145	2860 ± 270 (n = 21)	2720 ± 281	5150 ± 262 (n = 37)	5030 ± 291	0·98 ± 0·05 (n = 41)
Rabbit submaxillary	540 ± 79 (n = 21)	920 ± 155	6670 ± 417 (n = 18)	11650 ± 864	7090 ± 389 (n = 19)	12120 ± 542	0·61 ± 0·03 (n = 21)

Activity of respiratory enzymes (±S.E.) in normally innervated salivary glands of the cat and the rabbit. Units of enzyme activity are defined in the text.

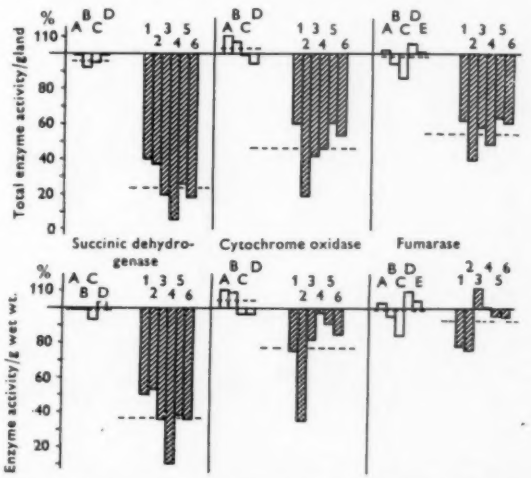

Fig. 1. The effects of preganglionic parasympathetic denervation on activities of cat submaxillary glands. The activity of the right gland is given as a percentage of that of the left gland. A, B, etc., refers to one unoperated cat; 1, 2, etc., to one animal in which the right gland was denervated. In all figures, □ = normal/normal; ▨ = denervated/normal; – – – = mean value.

Rabbit submaxillary gland. The results were very similar to those just described for the cat. There was a pronounced fall in the total activities of all three enzymes (Fig. 2). Enzyme concentrations were also decreased,

although there was a mean reduction in weight of 33 %. In the rabbit submaxillary gland the fumarase concentration was significantly decreased.

Cat parotid gland. Section of the post-ganglionic parasympathetic fibres

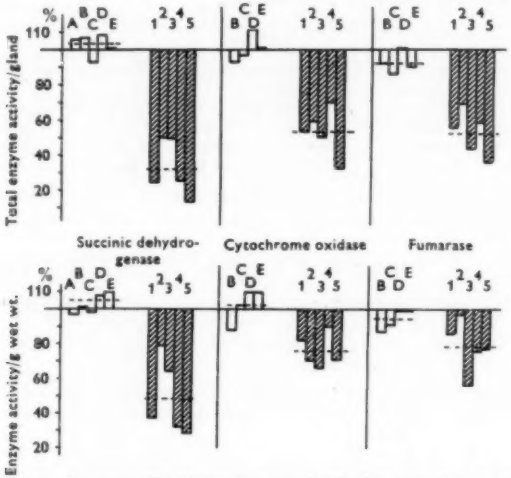

Fig. 2. The effect of preganglionic parasympathetic denervation on enzyme activities of rabbit submaxillary glands

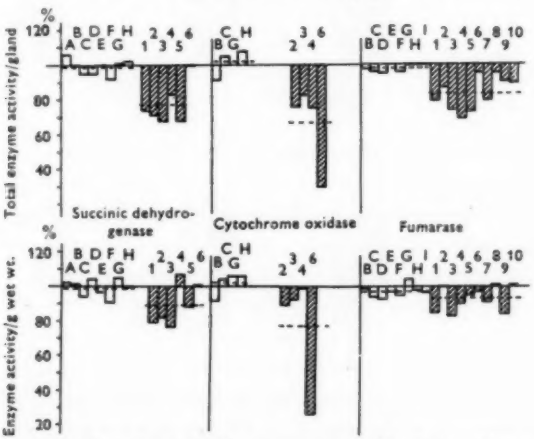

Fig. 3. The effect of post-ganglionic parasympathetic denervation on enzyme activities of cat parotid glands. The enzyme activities of the left (normal or denervated) gland are given as a percentage of those of the right gland.

to the parotid gland resulted in decreased enzyme activities (Fig. 3). The mean total activities in the denervated gland were decreased more than the weight of the gland (11 %) and thus the concentrations were also reduced.

The effect of preganglionic denervation was studied in three animals (not shown in Fig. 3), with an outcome very similar to that after post-ganglionic denervation. The mean total activities for succinic dehydrogenase, cytochrome oxidase and fumarase respectively were 59, 80 and 60 % of the normal and the corresponding values for enzyme concentrations were 73, 96, and 77 %. The weight loss after preganglionic denervation (17 %) was also very similar to that after post-ganglionic denervation (11 %). Similar weight reductions were found on larger groups of animals in earlier work (Strömblad, 1955*b*, 1956).

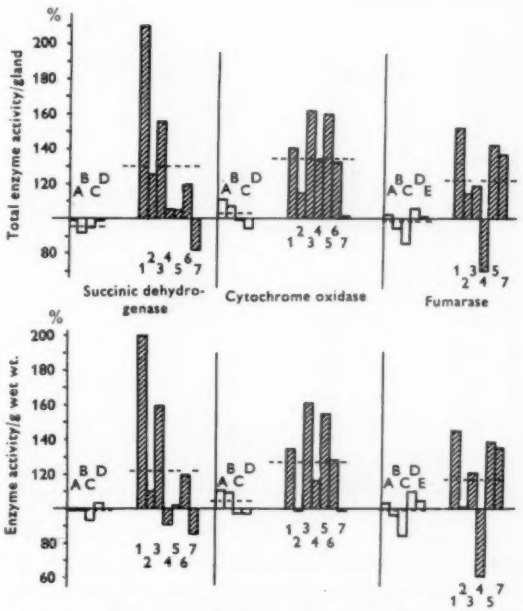

Fig. 4. The effect of excision of the superior cervical ganglion on enzyme activities of cat submaxillary glands

Sympathetic denervation

Cat glands. Excision of the superior cervical ganglion was followed by a rise of the same order in the total activity of all three enzymes in both parotid and submaxillary glands (Figs. 4, 5). The mean values for total activity ranged from 122 % of normal for fumarase in submaxillary glands to 145 % of normal for cytochrome oxidase in parotid glands.

The weight of the glands was increased after ganglionectomy. The weight of the right gland was calculated for each animal as a percentage of that of the left gland. In the operated animals with the right gland

denervated, the mean value ± S.E. was for parotid glands 109 ± 3·8 ($n = 8$) and for submaxillary glands 107 ± 2·6 ($n = 6$). These values differ ($P < 0·05$) from those found for unoperated animals (parotid glands 100 ± 1·2 ($n = 9$), submaxillary glands 99 ± 1·3 ($n = 5$)). Thus, ganglionectomy caused an increase in weight of both parotid and submaxillary glands in the cat; this increase, however, was less than the increase in total enzyme activity, so that the enzyme concentrations were also increased. The mean increase in enzyme concentration varied from 14% for fumarase to 32% for cytochrome oxidase, both of these values being found in the parotid glands.

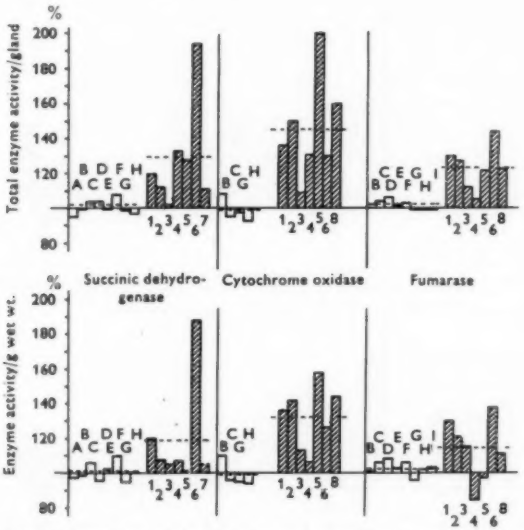

Fig. 5. The effect of excision of the right superior cervical ganglion on enzyme activities of cat parotid glands. The enzyme activities of the right (normal or denervated) gland are given as a percentage of those of the left.

Rabbit glands. Excision of the superior cervical ganglion in rabbits caused a decrease in enzyme activities. Figure 6 shows that there was a decrease in all three enzyme activities whichever way the results were expressed. The rabbit glands did not increase in weight (operated 96 ± 2·9 ($n = 6$); unoperated 100 ± 3·5 ($n = 5$)).

Time course of changes in enzyme activity

In order to get some information on the time course of the changes, the enzyme activity was estimated at various intervals after section of the chorda. The submaxillary gland of the cat was chosen for this study since this showed the most pronounced changes.

The glands from two cats were examined 24 hr after denervation, from two other cats after 4 days and from one cat after 7 days. After 4 days a decrease in weight was found (88 and 92 % of their controls). A decrease in succinic dehydrogenase activity was noted already after 24 hr (61 and 81 % of their controls). Cytochrome oxidase and fumarase activity was not reduced until the fourth day, when a definite decrease was found. After 7 days there was a further decrease of weight and enzyme activities, although it was in this animal less than that found for animals 3–5 weeks after denervation.

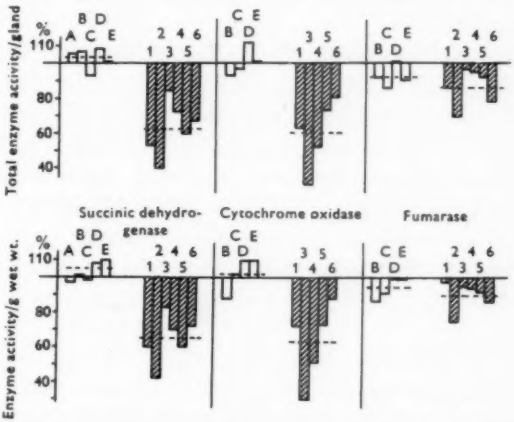

Fig. 6. The effect of excision of the superior cervical ganglion on enzyme activities of rabbit submaxillary glands.

DISCUSSION

After section of the chorda tympani the respiration of the submaxillary glands *in vivo* was found to be diminished (Strömblad, 1959). Similarly, experiments *in vitro* on chopped submaxillary glands of cats showed the maximal oxygen consumption to be reduced by division of the chorda; sympathetic ganglionectomy, on the other hand, increased the maximal oxygen uptake (Strömblad, 1957*a*). The changes in the activity of respiratory enzymes observed in the present investigation after denervation seem to afford an explanation of these findings.

The three enzymes studied are affected in the same way by denervation and can therefore be discussed together. Judging from previous experiments (Strömblad, 1956) the amine oxidase of the salivary glands can be included in this group. Cholinesterase, on the other hand, differs in its reactions to denervation in several respects from these enzymes. Extirpation of the superior cervical ganglion in cats causes an increase in

the activity of the respiratory enzymes and of the amine oxidase, but does not change that of the cholinesterase (Strömblad, 1957*b*). Furthermore, whereas the activity of the former enzymes decreases to the same extent after pre- and post-ganglionic parasympathetic denervation, the cholinesterase activity is reduced particularly by destruction of the ultimate neurone (Strömblad, 1955*b*). It is interesting to note in this connexion that the respiratory enzymes and the amine oxidase occur in the mitochondria (Cotzias & Dole, 1951; Hawkins, 1952), while the cholinesterase has been found mainly in the microsomes (Hagen, 1955; Toschi, 1959); in addition, it is assumed to be present outside the cell membrane (Koelle & Koelle, 1959).

It is possible that changes in the respiratory enzymes result from the inactivity of the glands brought about by denervation. The finding that the activity of various enzymes decreases after ligation of the salivary duct (Junqueira, 1951; Fernandes & Junqueira, 1953) supports this view. It is difficult, however, to explain in this way the increase in enzyme activity observed after sympathetic denervation of salivary glands in the cat. It may be due in some way to improved supply of blood following removal of the vasoconstrictor tone, but this explanation cannot be reconciled with the fact that such a change is not produced by the same procedure in rabbits.

Denervation is known to affect the weight of the salivary glands, and the present experiments suggest that this is in some way related to the changes in the activity of respiratory enzymes. Parasympathetic denervation reduces enzyme activity and weight. In cats both effects are more pronounced in the submaxillary than in the parotid gland. Removal of the sympathetic ganglion causes an increase both in weight and enzyme activity in cats; in rabbits there is no increase in weight and a reduction in enzyme activity. A change in the activity of the succinic dehydrogenase was found before any alteration in the weight could be detected, and this suggests that the effects on the weight are secondary to those on the activity of the respiratory enzymes. A similar explanation has been given for the effects occurring in skeletal muscle (Humoller, Griswold & McIntyre, 1951; Humoller Hatch & McIntrye, 1952).

SUMMARY

1. The activity of succinic dehydrogenase, cytochrome oxidase and fumarase was measured in normally innervated and in sympathetically or parasympathetically denervated salivary glands of the cat and rabbit.

2. Parasympathetic denervation caused a decrease in the activity of all three enzymes and a loss of weight of the glands.

3. Sympathetic denervation resulted in an increase in both the enzymic activities and the weight of cat glands, whereas a decrease in enzymic activities, unaccompanied by a gain in weight, was found in rabbit submaxillary glands.

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THE LATENCY AND FORM IN MAN OF THE OCCIPITAL POTENTIALS EVOKED BY BRIGHT FLASHES

By W. A. COBB and G. D. DAWSON

From the Department of Applied Electrophysiology and the Medical Research Council Neurological Research Unit, The National Hospital, Queen Square, London, W.C. 1

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The latency in man between a flash of light in the eyes and the electrical response in the occipital sensory cortex has been said to lie between 25 and more than 100 msec. Some uncertainty also exists about the polarity of the first occipital potential after the flash. Probably a large part of this uncertainty is due to the fact that the earliest parts of the responses are very small; this makes them difficult to identify amongst the relatively large spontaneous activity in the electroencephalogram (e.e.g.). Cobb & Morton (1952), using a superimposition technique (Dawson, 1947) found a clear surface positive wave with a latency of about 70 msec; this was preceded by much smaller deflexions which began perhaps as little as 22 msec after the flash. They were unable to decide whether these small early waves were of cortical origin, or whether they were produced by passive spread of current from the electroretinogram (e.r.g.). Since they coincided approximately with the 'b' wave of the e.r.g. this last possibility seemed quite likely. More recently Calvet, Cathala, Hirsch & Scherrer (1956), using a photographic averaging method, found a positive potential in the occipital region with a latency of 30–40 msec. Monnier (1957) has made measurements from records of 5 responses superimposed and gives the figure of 37·5 msec as the time from the flash to the first, positive-going, occipital potential in healthy subjects. Ciganek (1958), also using a superimposition technique, reports responses with an initial phase in which the occipital region becomes negative 25–35 msec after the flash. It is the purpose of this paper to present evidence, which has been obtained by more sensitive methods of recording, about the latency and form of the occipital potentials evoked by flash stimuli. This evidence, which has been reported in abstract elsewhere (Cobb & Dawson, 1956) supports the view that the early waves described by Cobb & Morton (1952) are of cortical origin and that the initial wave, when it can be recorded, is positive-going in the occipital region and has a latency of 20–25 msec.

METHODS

Eleven healthy adult subjects were used. They were examined lying face downwards with the head and thorax supported on soft pads, to secure the best relaxation of the neck muscles. In front of the face was an opal glass screen subtending an angle of 48° at the eyes. The surface of the screen contained no detail except when the effects of fixation were being studied. Then, a weak red light about 3 mm in diameter was placed in the middle of the screen. The back of the screen was illuminated by the discharge of a capacitor through a Xenon-filled flash tube and the front by the low level of ambient illumination in the darkened laboratory. The capacitor was charged to 2 kV and was usually of 16 μF, giving an energy in the flash of 32 J, but to alter the apparent brightness of the flash it was varied between 0·2 and 16 μF (0·4–32 J), the voltage being kept constant. With the 16 μF capacitor the peak luminance of the flash at the front of the opal screen, measured as described by Cobb & Morton (1952), was around $3{\cdot}4 \times 10^6$ cd/m^2 (10^6 ft-lamberts). The flash had a rapid rise and an approximately exponential decay with a time constant of 90 μsec, giving an integrated luminance with respect to time of 3×10^2 cd/m^2.sec. To reduce possible interaction between the responses to one flash and the later parts of the response to the flash preceding it, the rate of flashing was usually kept at one in 2 sec or slower. The occipital potentials were picked up with 1 cm diameter brine-soaked pads or with hypodermic needles blunted and spring-loaded on to the scalp (Dawson, 1954*b*). One electrode was placed on the external occipital protuberance (EOP) on the mid line; others at 3 cm intervals in front of it and also on lines 3 and 6 cm lateral to the mid line. An extra electrode was sometimes used 3 cm below the protuberance on the neck muscles in the mid line. Records were made from one or two pairs of these electrodes and the responses to between 55 and 220 flashes were averaged instrumentally (Dawson, 1953, 1954*a*). The polarity of connexion was such that when the electrode indicated in the figures by a continuous line became negative with respect to the electrode indicated by a broken line, an upward deflexion resulted in the record. The resistance-capacity coupled amplifiers used to feed the averaging machine had enough amplification to give a deflexion on the display cathode ray tube of 2 cm for an input of 2 μV. The high-frequency response of the recording system was limited by the sampling rate of the averaging device; each sample representing the mean potential over a period of 1·6 msec. For reasons dealt with elsewhere (Dawson, 1954*a*) the low-frequency response of the amplifiers was reduced as far as possible without gross distortion of the evoked potentials. The degree of distortion permitted is indicated by the calibration traces in Figs. 1 and 2 and corresponds to a coupling time constant of approximately 0·05 sec. The reduction of low-frequency response was carried out in one coupling only, to minimize phase shift. The other couplings in the amplifier had time constants of not less than 0·5 sec. The e.r.g. was recorded between a contact shell, with an electrode moulded into it, on the cornea and a pad on the forehead.

RESULTS

Relation of occipital responses to e.r.g.

When a subject is stimulated with flashes of the brightness used in these experiments and in the earlier ones of Cobb & Morton (1952, 1953) the e.r.g. recorded between cornea and forehead has an amplitude of about 200 μV. The initial phase of the occipital potentials recorded after the same flashes is rarely as big as 5 μV, and is usually much less. The recording methods used give great sensitivity but they can only discriminate against potentials which are not systematically related to the stimulus.

The possibility therefore exists that the small potentials picked up in the occipital region early after the flash are not part of the e.e.g., but are due to spread of current from the much larger e.r.g. That this is unlikely is shown when the e.r.g. and the occipital potentials are recorded simultaneously as in the records in Fig. 1*a*. Here 110 traces of the e.r.g. have been superimposed, trace 2, and the 110 occipital potential responses to the same stimuli have been averaged and recorded on the same time scale,

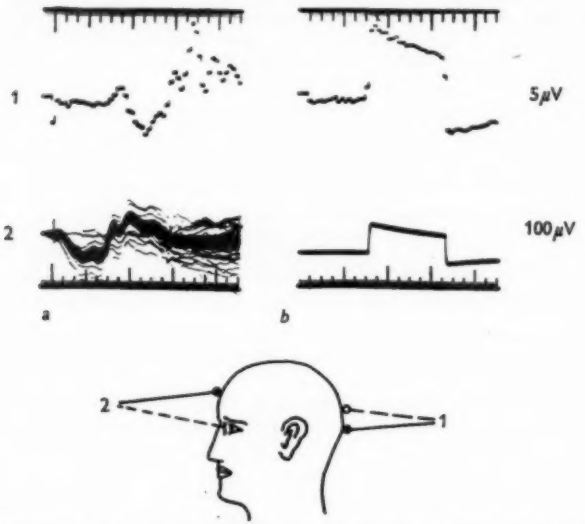

Fig. 1. The record in trace 1*a* is the average of the occipital potentials evoked by 110 bright flashes. In trace 2*a* are shown, superimposed, the retinal potentials produced by the same 110 flashes. In spite of the very high amplification used for trace 1 (voltage calibrations for the two traces are shown in *b*) there is no evidence of electrical spread to the occipital record of the retinal potentials. In these and all later records an upward deflexion indicates that the electrode shown connected by the broken line became positive with respect to that connected by the continuous line. The time scale shows intervals of 1, 5 and 20 msec and the time of occurrence of the stimulus is shown by the first large spike.

trace 1. The 'a' wave of the e.r.g. begins within 5 msec of the stimulus, reaches a maximum 13 msec after the stimulus and remains at about this level for 10 msec. During this time no significant deflexion appears in the occipital record. At 20–25 msec after the flash a series of considerable deflexions begins in the occipital record; none of these is synchronous with any potential having the same time course in the e.r.g. It therefore seems likely that spread of current from the e.r.g. plays no significant part in producing the occipital potential changes. Artifacts due to the heavy pulse of current through the lamp sometimes occur (Fig. 3*b*), but they

have not been found to last more than 1 or 2 msec after the flash was triggered. In the same way any photo-electric effects which occur disturb only the start of the trace and can be controlled by shading the electrode affected. Other sources of potential change systematically related to flashes which need to be considered are the ear and the auditory cortex. The records in Fig. 3*b* suggest that the clicks produced by the lamp are unimportant. In these records 110 sweeps were averaged while the lamp, although flashing and clicking, was covered up so that no light reached the subject. No detectable response occurs after the stimulus marker. However, when the experiment was repeated with the lamp uncovered the responses shown in Fig. 3*a* were recorded. It may fairly be concluded therefore that the initial phase of the occipital potential change represents events in the occipital cortex at about the time of arrival of the group of afferent impulses or shortly after this.

Latency of e.e.g. responses

The initial deflexion in the e.e.g. in Fig. 1 has an average amplitude of 1–1·5 μV. Although it was sometimes smaller than this, it was clear enough in nine of the eleven subjects for its start to be timed; in all these cases it lay between 20 and 25 msec after the flash. Superimposed on the rather variable slow waves which succeed the first e.e.g. deflexion is a series of faster waves. In nine subjects it was possible to measure from 4 to 7 of these waves and their period was found to be between 8 and 11 msec. In the responses recorded in Fig. 1 the mean period of the 5 waves is 10 msec, which corresponds closely with the period of the fast waves in the e.r.g. (Cobb & Morton, 1953). This may suggest that the fast waves in the e.r.g. represent the synchronized volleys of impulses which are known to leave the retina in some circumstances (Granit & Therman, 1935). Even if this is so, these waves are of little value in establishing the time of conduction from retina to cortex, because of the difficulty of identifying the corresponding waves of each series.

Location and polarity of e.e.g. responses

To avoid picking up the potentials from the eye, or from other parts of the brain, the occipital responses have usually been recorded from pairs of electrodes closely spaced in the occipital regions. Since either or both of such a pair of closely spaced electrodes may be affected by the change of potential during the response, no decision can be arrived at about the polarity of the evoked potentials until their source has been located. Attempts were therefore made to define the potential gradients in the occipital region during the responses, but this was found to be difficult, particularly in the anteroposterior direction. Reversal of the sign of the

gradients, indicating a maximum of activity, could usually be found when the electrodes had a separation of 6 cm, but attempts to define the point of reversal more accurately, by using electrodes at 3 cm intervals, sometimes led to the potential differences picked up during the initial phases of the responses being too small to detect, even when several hundred responses were averaged. In Fig. 2*a* records are shown from a subject in whom the potential gradients, though small, were well defined. Each trace in this figure represents the average of 220 single responses, and since only two averaging channels were available, the records were made in

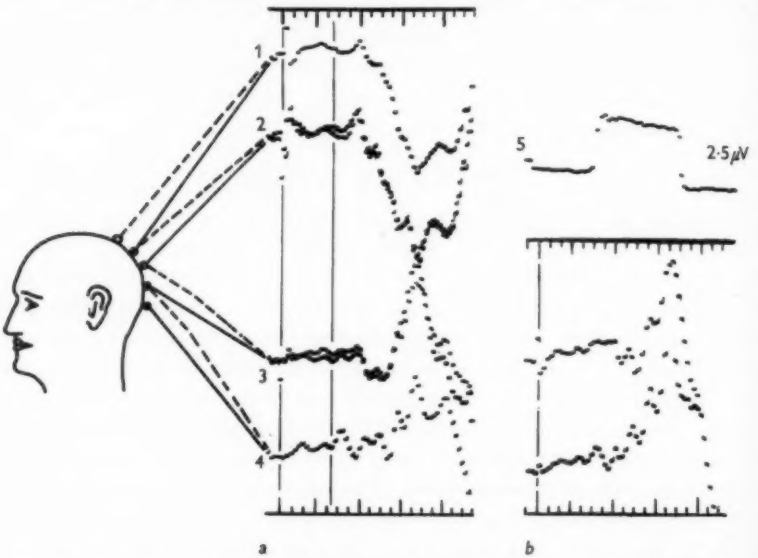

Fig. 2. The records in *a* show the potential gradients along the mid line in the occipital region and are the average of the responses to 220 flashes. The records were made in pairs, 1–2, 2–3 and 3–4. The duplicate records in traces 2 and 3 have been superimposed photographically and were made at intervals of 11 and 22 min. The records in *b* were made from similar electrode positions to those in traces 3 and 4 but more than 3 months later. The first vertical line shows the time of the stimulus and the second the start of the response. The time scale shows intervals of 5 and 20 msec. Trace 5 in *b* shows the voltage calibration for all channels.

pairs, 1–2, 2–3 and 3–4. The duplicate records in channels 2 and 3 have been superimposed photographically. The initial wave of the e.e.g. response is largest in trace 4, where it appears as an upward deflexion starting at 20–22 msec. It is absent in trace 3 and in traces 1 and 2 it appears as a small downward deflexion. This shows that during the first wave a region centred somewhere between the electrodes 3 and 6 cm from the EOP became positive with respect to other parts. From subject to subject this

location has varied slightly between the EOP and a point 6 cm in front of it, on the mid line. In records taken between an electrode on the mid line, 3 cm above the EOP, and others lateral to it, the later, larger waves in the responses appear symmetrical. The initial wave also sometimes appears symmetrical about the mid line; an example of this is shown in Fig. 3*c*, but more often when the central electrode is placed as near the mid line as can be measured from external landmarks, the first wave appears asymmetrical, as in the records in Fig. 3*a*. When the first wave is symmetrical in the records the sense of the deflexions in the traces shows that the mid-line electrode has become positive with respect to the lateral ones.

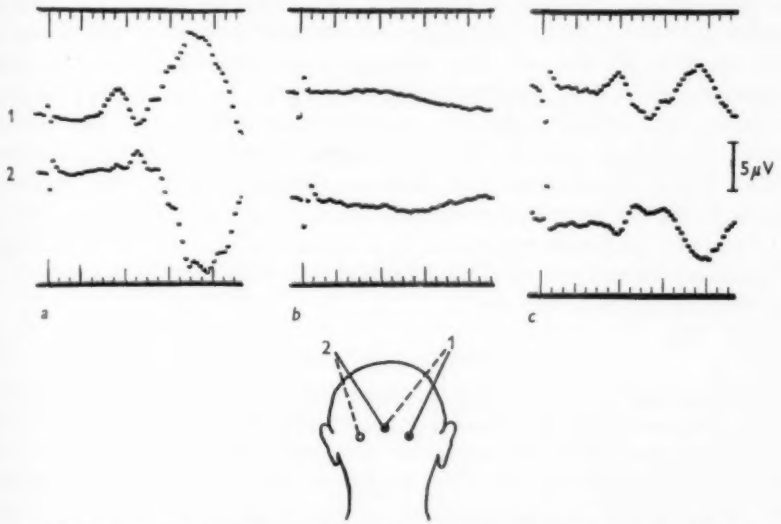

Fig. 3. Record *a* shows the average of the responses to 110 flashes with the recording electrodes across the mid line. In *b* the conditions were the same except that the lamp was covered up, showing that neither the click nor the electrical field produced by the discharge through the lamp causes any significant artifact at the time of the occipital response. The records in *c*, from another subject, where the initial wave is more symmetrical about the mid line, show that in the first wave of the response the electrode on the mid line becomes positive with respect to those lateral to it. The time scales show intervals of 1, 5 and 20 msec.

After the initial positive deflexion in the e.e.g. a series of other waves follows. From the records in Fig. 2 it can be seen that the relative sizes and forms of these later waves is greatly affected by small movements in position of the recording electrodes. However, of these waves the first three appear to have a high degree of constancy. Immediately after the initial positive deflexion is a negative-going potential with its trough at 35–45 msec. Succeeding this there occurs a large positive potential with

its peak at 60–70 msec which sometimes appears to have a potential distribution rather different from that of the initial positive wave. In the records in Fig. 2 the potential gradient over the occipital region during the second large positive wave reverses in sign at a point 6 cm in front of the EOP, whereas these records suggest that the potential gradients during the initial positive deflexion reverse in sign 1 or 2 cm more posteriorly than this. After this large positive deflexion there occurs a relatively much larger negative potential of the order of 5–10 μV in size. This late negative wave has its peak at round about 90–100 msec and it may be this which can sometimes be seen preceding the occiput-positive wave that appears in ordinary e.e.g. records taken during flash stimulation. All the records which are reproduced in this paper showed these four major components, the initial positive wave, the negative wave following it, the second positive wave and the later large negative wave. In two of the subjects, however, those from whom the records shown in Figs. 1, 2 and 4 were taken, the second positive wave is to some extent obscured by the fast ripple which appears with high flash intensities. The other two subjects, those giving the records shown in Fig. 3*a*, *c*, do not show these fast waves and the peak of the second positive wave is clearer. The recording methods available were not convenient for studying the later parts of the responses succeeding the first 100 msec.

Constancy of the responses

Under fixed conditions of stimulation, observation of single responses suggests that they vary in size considerably from one stimulus to the next. A great part of this apparent variability may be due solely to the background of spontaneous e.e.g. activity on which the evoked potentials are superimposed. In the averaged records, however, the main characteristics of the responses in one person remain little changed over periods of 20 min or more. This is shown in Fig. 2*a*, where the superimposed records of trace 2 were taken at an interval of 11 min and the pair in trace 3 at an interval of 22 min. The records taken at these intervals agree well and they give an indication of the degree of confidence which can be placed in the averaged responses obtained in this type of experiment. Over longer periods, such as that at which the records in Fig. 2*b* were taken, rather more than 3 months after those in Fig. 2*a*, it can be seen that, although the general character of the responses is unchanged, small variations in the relative sizes of the different phases occur. This order of variation in the form of the responses may easily have been caused largely by slight differences in electrode position between the two examinations.

Another factor altering both the relative sizes and latencies of the components of the responses is the brightness of the flash used. When

the flash strength is reduced, by reducing the size of capacitor which is discharged through the lamp, two effects occur. The latency of the initial waves of the response is increased and the amplitudes of all the waves in the response are reduced. In the record in Fig. 4*a* a capacitor of 16 μF (32*J*) was used and the initial positive deflexion in the e.e.g. response to the flash has a latency of 20–25 msec to its start. As the flash strength is reduced, little alteration appears down to a capacitor of 4 μF (8J; Fig. 4*b*) except that the size of the ripple of fast waves with a period

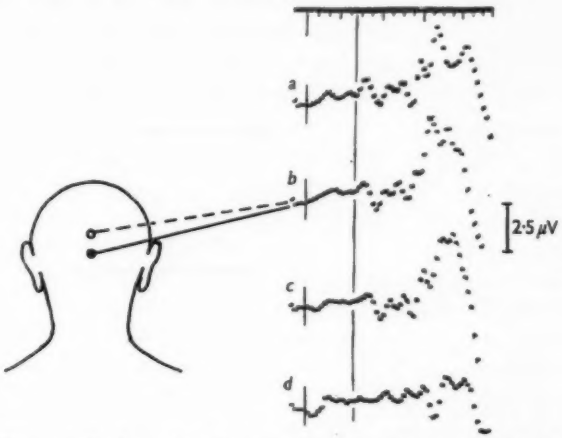

Fig. 4. The records show the effect on the occipital responses of reducing the apparent brightness of the flashes by reducing size of the capacitor discharged through the lamp. In *a* the capacitor was 16 μF, giving an energy in the flash of 32J; in *b*, 4 μF (8J); in *c*, 1 μF (2J) and in *d*, 0·2 μF (0·4J). The time of the stimulus is indicated by the first large spike in the time scale and the vertical lines through each record, and the start of the response in record *a* by the second vertical line. As the flash strength is reduced the latency increases and the amplitude of all parts of the response falls. The time scale shows 5 and 20 msec intervals. Average of 220 responses.

of 9–10 msec is reduced. A further reduction of the capacitor to 1 μF (2J; Fig. 4*c*) causes a distinct reduction in the size of the initial positive wave and an increase in the latency of its start to 25 msec. When the capacitor is reduced to 0·2 μF (0·4J; Fig. 4*d*) the flash appears to the subject to be much less bright and the latency of the initial positive wave increases to 30 or 35 msec; at the same time its amplitude is approximately half what it was in the top record, *a*, for which the 16 μF capacitor was used. No records are available to show how much of this increase in latency with a weaker flash is due to an increase in the retinal delay.

During some experiments it seemed that as the subject's attention was attracted to the auditory field, e.g. by conversion in the room, a change in

size of some of the responses occurred. Unfortunately this effect could not be maintained constant for a sufficiently long time for it to be recorded, but it was found that a change in the form and size of the response of the same type could be produced by visual fixation. The records in Fig. 5 give an example of this type of alteration. The upper record, *a*, shows the average response recorded when 110 flashes were delivered with the subject looking at the opal glass in front of the flash tube without any detail in the field and at a low level of steady illumination. In the lower record in Fig. 5*b* the conditions were the same except that the subject, throughout the time during which the flashes were being delivered, fixed his attention on a small red light in the middle of the opal glass. During this period of fixation the negative-going second wave, with its trough at 45 msec, was

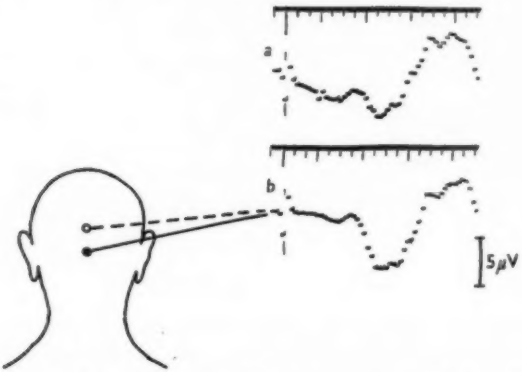

Fig. 5. The records show the average of the responses to 110 flashes. In *a* the opal screen in front of the lamp was blank. In *b* the subject fixed his gaze on a small red light in the middle of the screen throughout the period of stimulation. While the subject was looking at the red light the second, negative-going, wave of the occipital response was increased in amplitude. The time scale shows intervals of 1, 5 and 20 msec.

considerably increased in size. A difference of this sort between any two successive single responses is probably within the range of spontaneous variation, but since each record in the figure was the average of 110 responses, we believe the difference to be significant. At the same time as the negative wave increases in size, the duration of the initial positive wave is reduced, as though cut short by the development of the negative wave.

DISCUSSION

The results presented in this paper appear to resolve the doubts remaining from the previous work of Cobb & Morton (1952) about the latency of the occipital responses to flash stimuli. The records obtained

with averaging methods show clearly that a positive potential appears in the occipital region 20–25 msec after a bright flash stimulus. Comparison with a simultaneous record of the e.r.g. suggests strongly that the small positive potential in the occipital region is not due to a spread of potential from the eye. Other sources of artifact, such as electrical or photo-electric interference from the lamp, or potential changes from the auditory cortex caused by the noise of the discharge in the lamp, have also been excluded. It seems likely therefore that this initial positive wave in the occiput is analogous to the positive wave which may be found on the scalp over the somatic sensory receiving area after a peripheral stimulus and which has been taken to indicate the arrival of the afferent volley at the cortex. However, it must be noted that the positive potential evoked by somatic sensory stimulation may be preceded by a variable negative potential, usually less than 1 μV in size. The size of this negative wave varies both with the electrode position (Dawson, 1954*a*, Fig. 6) and with the particular peripheral nerve stimulated (Dawson, 1953, Fig. 12). No such initial negative potential has been recorded in these studies; probably if it occurs it is too small for the present recording methods to reveal it. Also in these experiments no sign has been seen of the brief initial spikes described by Marshall, Talbot & Ades (1943) when recording from exposed cat cortex and stimulating the optic nerve. The failure to record these spikes may be due either to the fact that they are much smaller when the stimulus is a flash to the eye rather than a shock to the optic nerve, as was shown by Marshall *et al.* (1943) or to the limited resolution of the recording method, both in time and in space because of the presence of the skull and scalp.

If it is accepted that these small potentials which we have recorded from over the occiput are in fact an indication of the arrival at cortex of the group of sensory impulses produced by the flash stimulus, or of the events shortly after this, then two chief points must be considered. In the first place Ciganek (1958, 1959) has stated that during the first wave of the evoked potential the occiput becomes negative with respect to other parts of the scalp, whereas we have found that during the first part of the response the occiput becomes positive. Secondly, Monnier (1957) gives the figure of 37·5 msec and Gastaut (1949) the figures of 40–45 msec for the latency between the stimulus and the start of the first wave of the response, whereas we have found a latency of 20–25 msec to the first wave. Both these differences probably arise from the very small size of the initial wave of the evoked potential. Cobb & Morton (1952) found that they could not be certain whether the first wave was present, even when large numbers of records were superimposed and the greatest possible care was taken to obtain muscular relaxation and to avoid other

artifacts which might confuse the record. With the averaging method, on the other hand, the occiput-positive first wave has been a constant feature of the responses, except when the recording electrodes have been symmetrically placed with respect to the peak of the potential gradients, as in trace 3 of Fig. 2*a*. It seems likely therefore that the deflexions taken as the first waves of the responses by Ciganek, Monnier and Gastaut were in fact the second or later waves. In several of the records shown by Ciganek (1959), notably Figs. 1 and 3 of that paper, there is a strong suggestion of a positive-going deflexion (downward in his display) preceding the negative deflexion which he takes as the first wave of the response. The start of this downward deflexion in his Fig. 1 appears to be at about 20 msec after the stimulus and its peak at about 25 msec; this would agree well with the start and the peak of the first positive wave that we record with the averaging technique. Probably therefore no conflict arises between our results and those of Ciganek. Brazier (1958), who used a cross-correlation technique, was also able to detect small occipital potentials starting some 20 msec after a flash stimulus.

The rather greater discrepancy between our results and those of Monnier (1957) and Gastaut (1949) may in part be due to a factor additional to the very small size of the initial positive potential. With the very bright flashes we have used the latency of the e.r.g. was 3–5 msec. It is difficult to compare this with the results of Monnier and Gastaut as they give no figure for the start of the e.r.g.; they do, however, give 30 and 40 msec for the start of the 'b' wave in their records. The start of the 'b' wave in our experiments is probably represented by the inflexion in the downgoing slope of the 'a' wave of the e.r.g. in Fig. 1. This occurs 10 msec after the stimulus. It seems therefore that the latency of the e.r.g. in our experiments may have been 10 or 20 msec less than in those of Monnier and Gastaut. How far such a reduction in latency of the e.r.g. is accompanied by corresponding reduction of the latency of the discharge in the optic nerve is not clear, but the over-all latency of the occipital responses in our experiments may have been not just *apparently* shorter, because of their small size, but really shorter than in the experiments of Monnier and Gastaut. Here again then it seems that there may be no real discrepancy between our results and those of Monnier and Gastaut. The differences merely emphasize the need for a complete specification of the conditions under which experiments of this kind are carried out.

Another factor in the stimulus situation which needs as full a description as possible is the amount of detail in the visual field and the degree of attention being paid to it. From the records shown in Fig. 5 it seems that concentration on a small red light in a previously blank field can cause an increase in size of the negative second potential in the response which

might be sufficient to raise the wave above the lower limit of detectability in superimposed records. It is not clear whether this increase in size of part of the response which accompanies a concentration of attention on the visual field represents an inverse effect to that described by Hernández-Péon, Guzmán-Flores, Alcaraz & Fernández-Guardiola (1957). They found that the occipital responses to flash stimuli in an unaesthetized cat were reduced when the animal's attention was attracted to another sensory field and they were able to show that there was a reduction in the size of the afferent volley at a subcortical level. That this effect, and the opposite effect also, may occur in man has been shown by Jouvet & Courjon (1958), who recorded, from an electrode implanted in the optic radiation, an increase in the size of the afferent volley when the subject concentrated on a visual stimulus. The only evidence available from our records on this point is the fact that when the negative second wave is increased, as in the record in Fig. 5*b*, the initial positive wave is not increased in size and may even be a little reduced. This may be slight evidence that the afferent volley has not increased in size, as the development of a large negative second wave in an evoked potential may cut short the positive initial wave. However, it has generally been the case in experiments on man that an increase in the strength of a somatic stimulus and an increase in the ascending volley in the peripheral nerve, although they sometimes lead to a disproportionate increase in the size of the negative wave of the evoked potential, have not caused this increase without an increase in the positive wave also (G. D. Dawson, unpublished observations). Since the positive wave was not increased, it may be suggested that this effect is more like those described by Dumont & Dell (1958) and Bremer & Stoupel (1959). They found that the size of the cortical response to stimulation of the optic nerve could be increased by stimulation elsewhere, and they showed that this effect was due more to a change in the state of the responding cortex than to an increase in the afferent transmission. An increase of the negative second wave of the somatic evoked potential without any increase of the positive first wave, or of the volley in the second sensory neurones in the afferent pathway, and thought to be largely intracortical, has also been described by Dawson, Podachin & Schatz (1959), but in these experiments the animals were anaesthetized and the effects of the anaesthetic have not yet been defined.

SUMMARY

1. The occipital evoked potential responses to bright flashes of light have been recorded by an integrating method which allows potential differences less than 1 μV to be detected.

2. Occipital potential changes of the order of 1–1·5 μV begin 20–25 msec after the flash. Comparison with the e.r.g. recorded simultaneously suggests that these occipital potentials are not due to current spread from the e.r.g. or other artifact.

3. From measurements of the latency of the occipital responses and the e.r.g. it is concluded that the transmission time from the retina to the cortex does not exceed 15 msec, though it could be less than this.

4. The potential gradients in the occipital region show that the initial wave in the e.e.g. is positive-going with respect to other parts of the scalp and has a maximum on the mid line, between 3 and 6 cm above the external occipital protuberance.

5. After the initial positive deflexion there occur a negative-going potential with its peak at 40–50 msec, a larger positive potential with its peak at 55–65 msec and a relatively large (5–10 μV) negative potential with its peak at 90–100 msec. This is followed by a series of waves with a period of about 100 msec.

6. With constant conditions the repeatability of the form of the responses is high over periods of 20 min. Over periods of months the repeatability is also good, though variations in the relative sizes of some of the components appear.

7. Reduction of the apparent brightness of the flash stimulus leads to a reduction in the size of the occipital responses and an increase in their latency.

8. Fixation on a detail of the visual field between flashes may lead to an increase in the second, negative, component of the response and a shortening of the initial positive deflexion.

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MECHANISM OF INCREASE IN NEGATIVE AFTER-POTENTIAL BY DICOPHANUM (DDT) IN THE GIANT AXONS OF THE COCKROACH*

BY T. NARAHASHI AND T. YAMASAKI

From the Laboratory of Applied Entomology, Faculty of Agriculture, University of Tokyo, Japan

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Attempts have already been made to see whether dicophanum (DDT) causes changes in after-potentials in nerve. In crab nerve there was only a slight increase in negative after-potential (Shanes, 1949*b*), whereas no change was observed in squid and frog nerves (Shanes, 1949*a*, 1951). On the other hand, it was found in our previous studies that DDT causes a marked increase and prolongation of the negative after-potential in cockroach nerve (Yamasaki & Ishii, 1952; Yamasaki & Narahashi, 1957*b*, *c*, *d*). This effect of DDT, together with its unstabilizing action, is regarded as the most important feature of the toxic action of DDT (e.g. Roeder & Weiant, 1948; Welsh & Gordon, 1947; Yamasaki & Ishii, 1954). However, there is no adequate evidence to explain the increase in negative after-potential caused by DDT.

As in squid giant axons (Frankenhaeuser & Hodgkin, 1956) and in non-myelinated nerve fibres of the rabbit (Greengard & Straub, 1958), the negative after-potential of normal cockroach giant axons has been demonstrated to be explicable by an accumulation of the released potassium ions in the immediate vicinity of the nerve membrane (Narahashi & Yamasaki, 1960). The present paper gives the results of experiments whose aim was to elucidate the mechanism of the increase in negative after-potential brought about by DDT.

METHODS

The giant axons of the American cockroach, *Periplaneta americana* L., were used throughout the experiments. The methods of stimulation and recording were essentially similar to those described in our previous reports (Yamasaki & Narahashi, 1959*a*, *b*; Narahashi & Yamasaki, 1960). Stimulation was effected by a pair of silver wire electrodes, while recordings were made by a micro-electrode inserted into an axon. When polarizing currents were to be applied to the axon, a Wheatstone bridge circuit was employed, which enabled a single micro-electrode to be used both for polarization and recording. The bathing solution was changed by the method described previously (Narahashi & Yamasaki, 1960).

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The Ringer's solution used was the same as that described in our previous papers (Yamasaki & Narahashi, 1959*a*; Narahashi & Yamasaki, 1960). DDT-Ringer's solution was prepared by introducing a small amount of ethyl alcohol containing pure DDT into Ringer's solution. The final concentration of DDT was 10^{-4} M, and that of the alcohol was 0·5%. This concentration of alcohol was found to have no effect on the axon in any respect. All the experiments were conducted at room temperatures ranging from 16·5 to 26·5° C.

RESULTS

Changes of resting and action potentials in the course of DDT poisoning

The action potentials before and after treatment with DDT are shown in Fig. 1. After the normal bathing solution had been replaced by a Ringer's solution containing DDT at a concentration of 10^{-4} M, no noticeable change was observed in resting and action potentials for a period of several

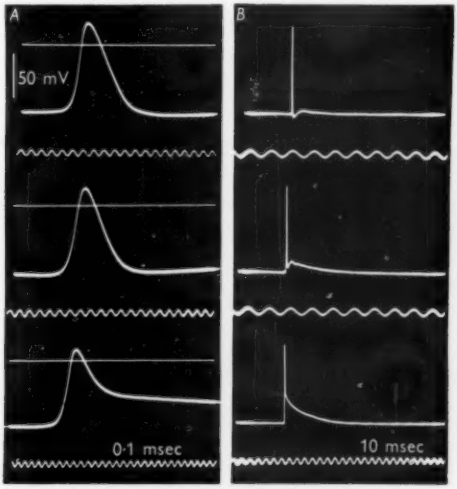

Fig. 1. Records showing changes in action potential after treatment with 10^{-4} M DDT. *A*, from top to bottom, before, 38 min after, and 90 min after treatment with DDT. The horizontal lines indicate zero potential level. *B*, as in *A*, but with slower sweep. Temperature 21·5° C.

minutes. However, after 10 min or so the negative after-potential began to grow progressively. The positive phase decreased as the negative after-potential increased. After some time, usually 15–30 min after starting treatment with DDT, the axon reached a state in which a single shock could give rise to a repetitive response (Fig. 2). With the further advance of time the negative after-potential grew larger still and the repetitive response ceased. The negative after-potential continued to grow more and more, and the positive phase could be seen as a slight dip between the spike

potential and the negative after-potential. The positive phase eventually disappeared, so that the spike potential terminated directly in the negative after-potential. The initial height of the negative after-potential finally attained a maximum value of about 30 mV or more, which stayed unchanged for some period of time. At this stage of poisoning a slight positive after-potential or undershoot sometimes followed the negative after-potential. Up to this stage the spike phase underwent little change; details will be described later. Thereafter, the rate of fall of the spike potential slowed considerably. With the further advance of time the negative after-potential began to decrease. The time of onset of this decrease varied considerably from fibre to fibre, being 3 hr or more after starting treatment with DDT. Conduction block was never observed during experiments, which were extended over 3–5 hr. The resting potential underwent little change throughout. On washing with normal Ringer's solution, these effects of DDT were found to be irreversible.

Thus, the time course of DDT poisoning can be divided into at least four stages: (1) from the beginning of the increase in negative after-potential to the end of repetitive discharge; (2) from the end of repetitive discharge to the maximum negative after-potential; (3) from the beginning of marked slowing of the rate of fall of the spike potential to the beginning of marked decrease in negative after-potential; and (4) the stage of marked decrease in negative after-potential. Unless otherwise stated, the present paper describes the results of experiments on stages (1) and (2), which are thought to be indicative of the primary action of DDT. Experiments on stages (3) and (4), which seem to some extent to be secondary to stages (1) and (2), will be described elsewhere.

Nature of repetitive response induced by DDT

Figure 2 shows records of the action potential taken before, during and after the state of repetitive response under the influence of DDT. Transition from the state where there was only a single response to that where there was a repetitive response occurred abruptly when the negative after-potential had increased to some extent. There appeared to be no intermediate state. The repetitive response started at a frequency of 200–400/sec and ended abruptly after a period of 30–70 msec. The interval between the first and second impulses was relatively large, between the second and the third impulses it was shortest, and it gradually grew towards the end of the train. On the other hand, transition from the state of repetitive response back to the state of single response was not always abrupt. This transition was invariably accompanied by a further increase in the negative after-potential. On some occasions the interval between the spikes was prolonged and the number of spikes decreased until the

response became single. In other cases, however, the state of single response was established abruptly. After the repetitive period, small potential changes were frequently seen on the declining phase of the large negative after-potential, as is shown in Fig. 2. These small potentials were found from close inspection to be damped oscillations starting at the peak of the negative after-potential and ending with completion of repolarization. The oscillations later decreased in magnitude as the negative after-potential increased further with the advance of time.

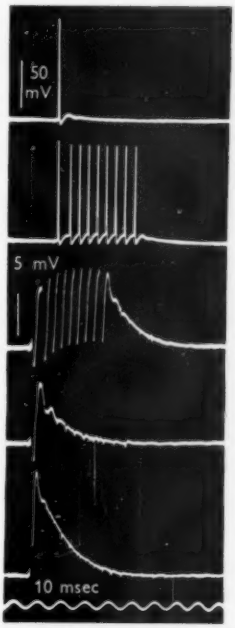

Fig. 2. Records of the action potential induced by a single stimulus before, during and after the repetitive stage in the course of DDT poisoning. From top to bottom, 20, 22, 26, 26·5 and 34 min after treatment with 10^{-4} M DDT. Temperature 21·5° C.

Repetitive response could also be induced in the DDT-poisoned axon by a long-lasting cathodal current. The normal cockroach giant axon did not give rise to a repetitive response even when a long-lasting cathodal current of suprathreshold intensity was applied. However, when the axon reached a state in which a single shock could give rise to a repetitive response, the long-lasting cathodal current was effective in producing a repetitive response (Fig. 8).

Effect of DDT on the configuration of the spike potential

The magnitude of the action potential was little affected by DDT (Fig. 1). Although there was a slight tendency for the action potential to decrease in height during the course of long-term experiments which extended to stages (3) and (4), it seems unlikely that this was due to any direct effect of DDT. The active membrane potential was probably kept almost constant throughout, because, as already mentioned, the resting potential underwent little change.

Graphical analyses of records of the action potential, such as those shown in Fig. 1*A*, revealed that the maximum rates of rise and fall were only slightly decreased by DDT, the effect on the latter being greater than that on the former. This result suggests that DDT has little effect on the mechanism by which the spike phase of the action potential is generated. However, during later stages of poisoning (stages (3) and (4)), the rate of fall of the action potential decreased so much that discrimination between the spike and the negative after-potential became difficult.

Records of trains of impulses at varying frequencies are illustrated in Fig. 3*A*. Although the DDT-poisoned axon could respond to repetitive stimulation at a frequency as high as 200–300/sec, the spike height was greatly reduced for all impulses except the first one. In the normal unpoisoned axon the spike height was also reduced during a train of impulses, but to a much less extent. This may imply that the axon is in a partially inactivated state after the first impulse.

Effect of DDT on negative after-potential

Negative after-potential following a single impulse. The time course of the decay of a negative after-potential following a single impulse was plotted on a logarithmic scale against time, as is shown in Fig. 4. Before treatment with DDT the decay could be expressed as a single exponential term. After treatment with DDT, however, the decay was not a single exponential function. The initial brief period of the decay could be described by an exponential term, which finally terminated in a faster phase. Since the height of the negative after-potential changed progressively during the course of DDT-poisoning, measurements were made when the maximum value for the negative after-potential was achieved in each series of experiments. The time constant for the initial phase thus estimated was between 14·6 and 40·4 msec in different axons, with a mean of 24·3 msec. This mean time constant is much longer than that of the normal unpoisoned axon, which has been estimated as 9·2 msec in our previous report (Narahashi & Yamasaki, 1960). The terminal fast phase also declined exponentially, with

a time constant ranging between 3·4 and 34·1 msec, and a mean of 14·7 msec.

Negative after-potential following a train of impulses. Figure 3*B* shows records of the negative after-potential at various frequencies of stimulation, The after-potential following a single impulse was already large, and there was only a small additional build-up during a train of impulses. It seemed

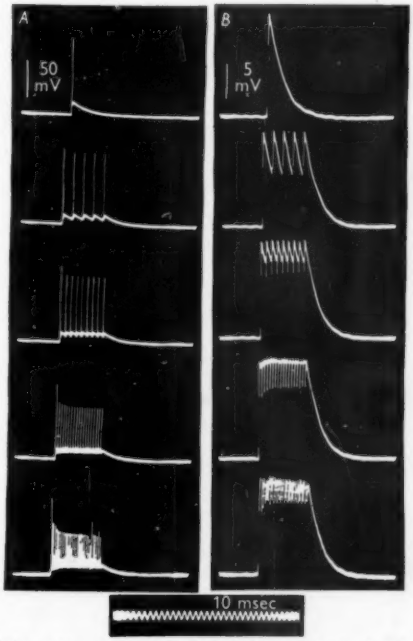

Fig. 3. Records of the action potentials induced by a single shock and by repetitive stimulation at various frequencies. These records were taken 50–62 min after starting treatment with 10^{-4}M DDT. *A*, from top to bottom, single stimulus, 50/sec, 100/sec, 200/sec and 300/sec. *B*, as in *A*, but with higher amplification. Temperature 18° C.

at first sight that the time course of the falling phase of the negative after-potential was independent of the stimulus frequency. To investigate this point further the following analyses were made.

Figure 5 shows the time course of the decay of the negative after-potential after a train of impulses at frequencies of 50 and 200/sec. The initial phase decayed approximately exponentially with a time constant close to that for the decay after a single impulse. However, there was a tendency for the declining phase to terminate in a very slow component. The initial phase continued longer than in an unpoisoned axon, but transition from the initial phase to the terminal phase occurred at a

comparable potential level in both the poisoned and unpoisoned axons. On the other hand, there was little sign of the terminal fast phase which was always observed in the negative after-potential after a single impulse.

Summarizing the various declining phases of the negative after-potential, the following patterns can be discriminated:

a, unpoisoned axon, single impulse, single fast phase;

b, unpoisoned axon, train of impulses, initial fast phase and terminal very slow phase;

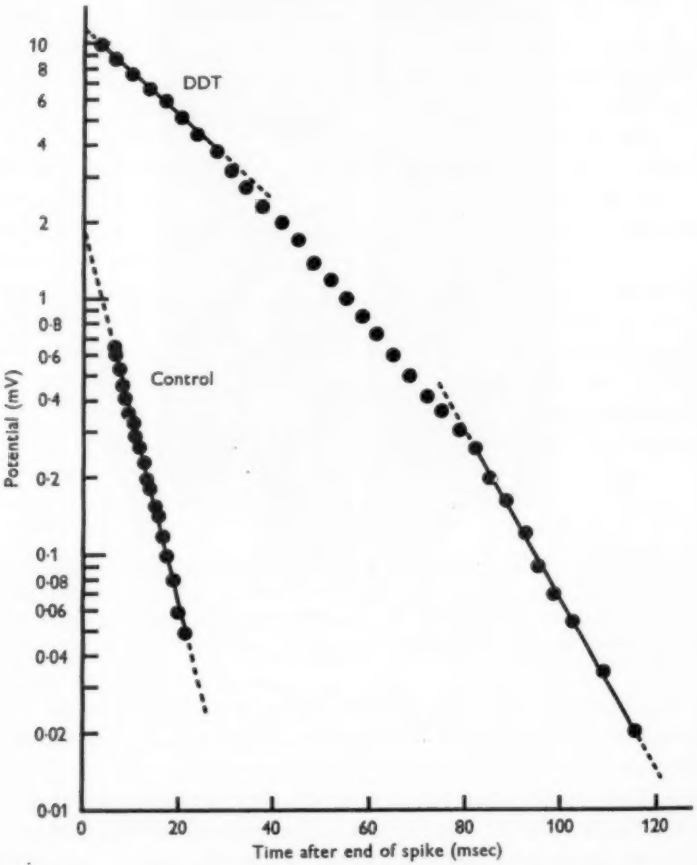

Fig. 4. Time course of the decay of the negative after-potential following a single impulse before and 53 min after starting treatment with 10^{-4} M DDT. The straight lines were drawn through the points by eye. Their time constants were: control, 5·8 msec; DDT, initial phase, 24·5 msec; DDT, terminal phase, 12·7 msec. Temperature 19° C. Semi-log scale.

c, DDT-poisoned axon, single impulse, initial slow phase and terminal fast phase;

d, DDT-poisoned axon, train of impulses, initial slow phase and terminal very slow phase.

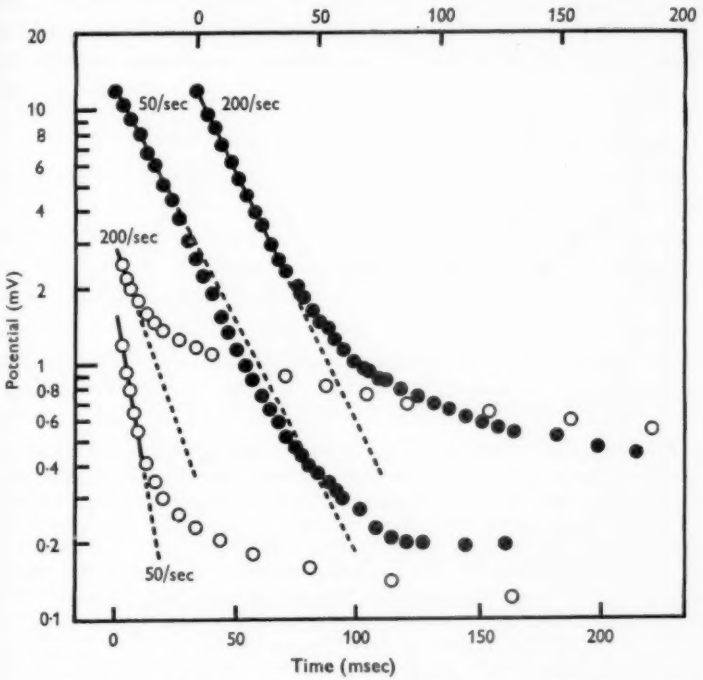

Fig. 5. Time course of the decay of the negative after-potential following a train of impulses at 50 and 200/sec before and 55 min after starting treatment with 10^{-4}M DDT. The open circles represent the potential before treatment, and the filled-in circles after treatment. The ordinate indicates the potential in mV on a logarithmic scale. The abscissae are the time in msec after the end of the last impulse in the train, the upper scale applying to 200/sec (after treatment) and the lower scale to the others. The straight lines were drawn through the points by eye. Their time constants were: before treatment, 50/sec, 8·6 msec; 200/sec. 16·3 msec; after treatment, 50/sec, 24·1 msec; 200/sec, 22·1 msec. The same axon as in Fig. 4. Temperature 19° C.

If the mechanism for the production of the terminal very slow phase of *b* is unimpaired by DDT, and if the mechanism for the production of the fast phase of *c* is also operative when the axon is excited repetitively, these two mechanisms should partly cancel each other when the DDT-poisoned axon is stimulated repetitively. This may result in the appearance of the prolonged initial phase in the repetitively stimulated DDT-poisoned axon.

Addition of negative after-potential during a train of impulses. There are two alternative explanations for the increase in negative after-potential caused by DDT. Two important features of DDT poisoning, which are the increase in the negative after-potential and the appearance of the terminal fast phase in the decay of the negative after-potential, may imply that the mechanisms by which the falling phase of the action potential is normally accelerated are delayed or partly suppressed by treatment with DDT.

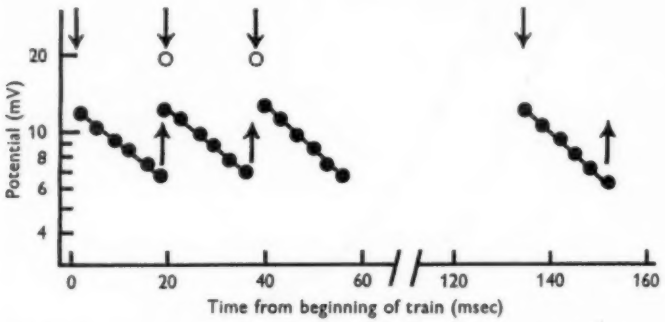

Fig. 6. Time course of the decay of the negative after-potential during a train of impulses at 54/sec, 53 min after starting treatment with 10^{-4}M DDT. The first, second, third and eighth impulses are illustrated. The filled circles are the measured points. The straight lines were drawn through the points by eye. Their time constants were: first, 29·9 msec; second, 29·6 msec; third and eighth, 26·4 msec. The open circles represent the potential at the end of the second and third spikes, calculated by adding the initial height of the first negative after-potential to the after-potential remaining at the instant when the second and third spikes arise. The upward arrows indicate the start of the spikes, and the downward ones the end of the spikes. The same axon as in Figs. 4 and 5. Temperature 19° C. Semi-log scale.

Alternatively, such an increase in negative after-potential could be caused by an increased accumulation of potassium near the membrane during activity.

If the second explanation were correct, the negative after-potentials in poisoned axons would add in a linear manner, as in unpoisoned axons. To make this argument clear, the successive negative after-potentials during a train of impulses at a frequency of 54/sec were plotted on a logarithmic scale against time, as illustrated in Fig. 6. The initial height of the second negative after-potential, estimated by adding what remained of the first after-potential at the moment when the second spike arose to the initial height of the first negative after-potential obtained by extrapolation, was $6{\cdot}6+12{\cdot}4 = 19{\cdot}0$ mV, as against the observed value of 12·5 mV. The calculated initial height for the third negative after-potential was $6{\cdot}3+12{\cdot}4 = 18{\cdot}7$ mV, as against the observed value of 12·7 mV. Further,

the amount by which the eighth impulse in a train declined in the same period as the stimulus interval was not equal to the contribution of a single impulse, the former value being 12·4 − 6·0 = 6·4 mV as against the latter of 12·4 mV. Hence it is concluded that the increase in negative after-potential caused by DDT does not arise from a greater amount of potassium release during activity. The small build-up of the negative after-potentials during a train of impulses, however, is attributable to the accumulation of released potassium.

Effect of DDT on the rectifying property of the membrane

The delayed rectification of the cockroach nerve membrane has already been demonstrated in one of our previous reports (Yamasaki & Narahashi, 1959*b*). Since the action potential of the cockroach giant axons has been shown previously (Yamasaki & Narahashi, 1959*a*) to be accounted for by the ionic theory (Hodgkin, 1957), the rectification can be interpreted on the assumption that the potassium conductance, a rise of which is responsible for the falling phase of the action potential, is kept increased during a flow of cathodal current. It was suggested in the preceding section that the increase in negative after-potential caused by DDT might arise from a delay or partial suppression of the mechanism by which the falling phase of the action potential is accelerated. The falling phase of the action potential is caused by an inactivation of the sodium-carrying system, which leads to a decline of sodium conductance, and by a rise in potassium conductance. Hence, if the rise in potassium conductance during activity was delayed or partly suppressed by DDT, it would follow that the delayed rectification would be affected.

Figure 7 shows records of the electrotonic potential from a normal axon induced by equal and opposite long-lasting currents. In accordance with our previous observation (Yamasaki & Narahashi, 1959*b*), the delayed rectification became apparent when the intensity of current exceeded a certain level. There also appeared a graded response with subthreshold cathodal currents. It should be noted that the decay of the catelectrotonic potential was faster than that of the corresponding anelectrotonic one, and terminated in a positive phase. The faster decay and the positive phase can be interpreted as indicating that there is an appreciable rise in potassium conductance during a flow of cathodal current.

Records of the electrotonic potential under the influence of DDT are illustrated in Fig. 8. The axon was in a state of repetitive response when these records were taken. The bottom record was taken with a single shock applied to the end of the nerve preparation by external electrodes. The top record is an electrotonic potential induced by a just subthreshold cathodal current, and the pair of records in the middle are for equal and opposite

threshold currents. It will be seen that the graded potential, which rose and fell as quickly as normal, was followed by a gradual rise in potential which then declined to a lower steady state after attaining a summit. On cessation of cathodal current the electrotonic potential decayed slowly without any sign of positive overshoot. A similar time course for the catelectrotonic potential was also seen when a threshold current was applied. Thus it became clear that in the DDT-poisoned axons the rectification occurred on application of cathodal current with a delay much

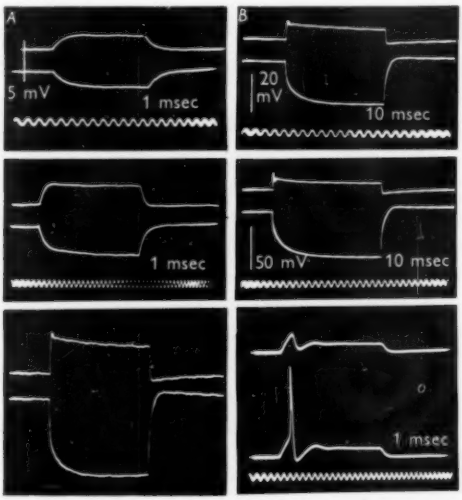

Fig. 7. Records of the electrotonic potential induced by equal and opposite currents with various intensities in a normal unpoisoned axon. The intensity of current was increased in steps from the top of column *A* to the bottom of column *B*. Upper tracing in each record shows catelectrotonic potential, and lower tracing anelectrotonic potential, except for the bottom record of *B* where just subthreshold and threshold catelectrotonic responses are shown. The time marker of the top of *B* also applies to the bottom of *A*. Temperature 20° C.

greater than normal. The degree of rectification was also depressed by DDT. This is undoubtedly responsible for the slower decay and the absence of the positive phase following break of cathodal current. The slower decay of the catelectrotonic potential was also observed in our previous study which was made with external electrodes (Yamasaki & Narahashi, 1957*d*).

These results strongly support the view that the rise in potassium conductance during activity was delayed and partly suppressed by DDT. Despite this change in delayed rectification the initial falling phase of the spike potential undergoes little change. It seems therefore reasonable to assume that DDT does not inhibit the inactivation process occurring

during the falling phase of the action potential, at least in the initial stages of poisoning.

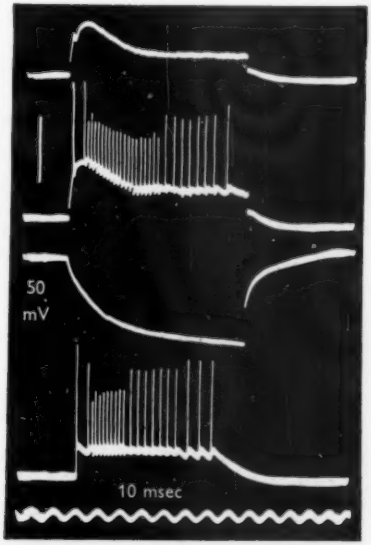

Fig. 8. Responses of a DDT-poisoned axon to long-lasting currents and a single shock. 41–44 min after starting treatment with 10^{-4} M DDT. From top to bottom, catelectrotonic potential induced by a just-subthreshold current; catelectrotonic potential with superimposed repetitive spikes induced by a threshold current; anelectrotonic potential with same threshold current; train of impulses induced by a single shock. Temperature 26·5° C.

Effects of potassium and calcium on the DDT-induced negative after-potential

Effect of changing the external potassium concentration. Figure 9 shows records of the action potential taken in 0, 3·1 and 10·0 mM-K solutions each containing DDT at a concentration of 10^{-4} M. The DDT-induced negative after-potential was very effectively depressed by high potassium solution. In a potassium-free solution it was greatly augmented and a plateau appeared. A small positive after-potential followed the plateau. There occurred a fall of resting potential when the potassium concentration was raised and a rise when it was lowered, as in unpoisoned axons. These effects of potassium were completely reversible upon washing with normal Ringer's solution. It should be noted that the action potential from the axon bathed in a potassium-free DDT solution resembles the cardiac action potentials from mammals in that there appears to be a marked plateau phase.

The delay and suppression of the rectification by DDT were also observed in potassium-free solutions.

Effect of changing the external calcium concentration. As is shown in Fig. 10, when an axon had been treated with 10^{-4} M DDT and was in a state giving rise to an increased negative after-potential, a rise in external calcium concentration from 1·8 to 18 mM caused a further increase in

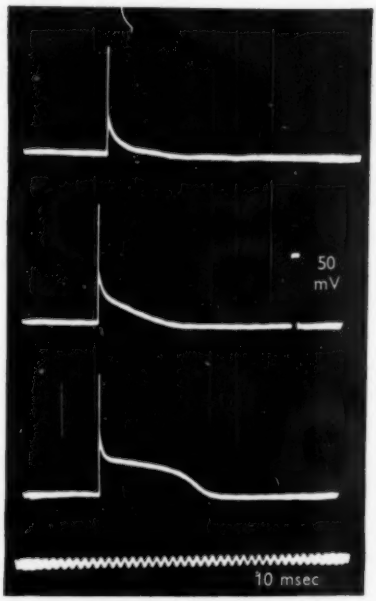

Fig. 9. Effect of changing the external potassium concentration on the action potential of the DDT-poisoned axon. These records were taken 85–112 min after starting treatment with 10^{-4} M DDT. Top record, 10 mM-K; middle record, 3·1 mM-K (normal); bottom record, 0 mM-K. Voltage calibration is given by a brief square pulse in the middle record. Temperature 26·5° C.

negative after-potential. The spike potential in the DDT-poisoned axon was affected in the same way as in an unpoisoned axon (Narahashi & Yamasaki, 1960), the maximum rate of rise being slowed down. These effects of calcium were completely reversible on washing with normal Ringer's solution.

Figure 11 shows records of repetitive activity as induced by a single stimulus in DDT-Ringer's solutions containing 1·8 mM (normal) and 18 mM-Ca. Two points are worth noting: (*a*) in spite of a tenfold increase in [Ca], the DDT-poisoned axon was still able to fire repetitively in response to a single stimulus. (*b*) The spike height was reduced progressively during a train of impulses in 1·8 mM-Ca, whereas it was little affected in 18 mM-Ca.

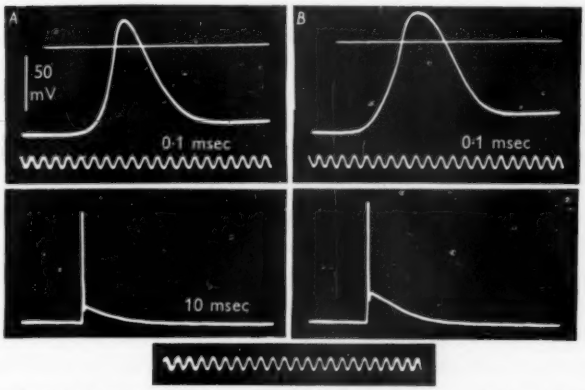

Fig. 10. Effect of changing the external calcium concentration on the action potential of the DDT-poisoned axon. These records were taken 69–78 min after starting treatment with 10^{-4} M DDT. The horizontal lines indicate zero potential level. *A*, 1·8 mM-Ca (normal); *B*, 18 mM-Ca. Temperature 19° C.

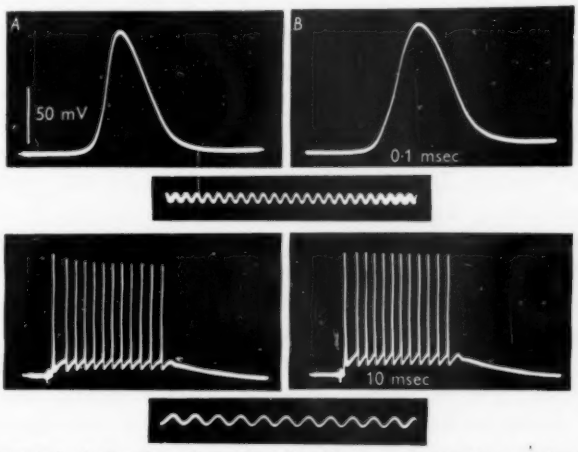

Fig. 11. Effect of changing the external calcium concentration on the train of impulses induced by a single stimulus in a DDT-poisoned axon. *A*, 1·8 mM-Ca (normal); *B*, 18 mM-Ca. Temperature 21·5° C.

DISCUSSION

It seems probable that the moderately increased negative after-potential is partly responsible for the repetitive discharge, because the state of repetition is first established when the negative after-potential has grown to some extent. As suggested for the production of repetition in myelinated nerve fibres by veratrine (Tasaki & Mizuguchi, 1948), and in

muscle fibres by tetraethylammonium (TEA) (Hagiwara & Watanabe, 1955), re-stimulation by an increased negative after-potential can be regarded as one of the mechanisms involved. However, the existence of other important mechanisms is suggested by the fact that normal unpoisoned axons of the cockroach do not give a repetitive response on application of a long-lasting cathodal current (Yamasaki & Narahashi, 1959*b*). Hence, it can be stated that DDT establishes a condition in which a repetitive response arises from any long-lasting depolarization, such as that provided by an increased negative after-potential.

It is interesting to note in this connexion that damped oscillations are observed on the decay of the negative after-potential immediately after the repetitive period is over. It seems reasonable to assume that the above-mentioned condition, which favours repetition when the negative after-potential increases, is related to an augmentation of oscillatory potentials. Thus, if the tendency for the axon membrane to set up oscillatory potentials in response to cathodal currents is increased by DDT, it follows that the increased and prolonged negative after-potential following a spike generates large oscillatory potentials, which are able to give rise to action potentials if they are of sufficient amplitude. The second impulse likewise sets up the third impulse and so on. With the advance of time the state of repetition is suppressed as described before. At this time the oscillatory potentials are so decreased in amplitude that they fail to give rise to action potentials. They can be observed as damped oscillations superimposed on the declining phase of the negative after-potential. In view of these considerations it may be stated that the induction of repetition by DDT which is observed in the initial stage of poisoning can be interpreted as being due to the two factors, i.e. moderately increased negative after-potential, and augmented oscillatory potentials.

Oscillations of membrane potential have received considerable attention (Arvanitaki, 1939, 1943; Cole & Curtis, 1941; Cole, 1941; Hodgkin & Rushton, 1946; Shanes, 1949*a*; Sjodin & Mullins, 1958). Cole & Curtis (1941) and Hodgkin & Rushton (1946) have shown that oscillatory property of the membrane depends on the apparent inductance, resistance and capacity of the membrane. Thus the damped oscillations observed during a flow of cathodal current in *Carcinus* axons were attributed to a decrease in membrane resistance upon depolarization (Hodgkin & Rushton, 1946). This explanation seems to be applicable to the present case; a decrease in membrane resistance caused by an augmented negative after-potential would cause oscillations, if the three parameters of the membrane property were altered by DDT in such a manner as to favour oscillations. However, since no data are available for alteration of the membrane constants by DDT, it is unwise to speculate further. It should be added that

Shanes (1949*a*) observed the after-potentials of the normal squid giant axon to be oscillatory.

Various hypotheses have been put forward for the mechanism of action of DDT on nerve. Welsh & Gordon (1947) and Gordon & Welsh (1948) carried out a series of experiments on the repetitive response of crayfish axons under the influence of DDT and various concentrations of calcium. They found that in the DDT-treated axon a single stimulus gives rise to a train of impulses. This effect was accelerated by low [Ca], and was similar to that produced by low [Ca]. They concluded that DDT is adsorbed at or near the axon surface and acts as a barrier layer which hinders the normal reaction of calcium ions with the surface.

In our previous studies the effects of DDT, potassium and certain metabolic inhibitors on the resting and action potentials of the cockroach nerve were examined with external electrodes (Yamasaki & Narahashi, 1957*a*, *d*). Unlike potassium and metabolic inhibitors, DDT had little or no effect on the resting potential and impulse conduction, though a marked increase in negative after-potential was caused. It was suggested that DDT affects the nerve function by changing the ionic permeability of the nerve membrane.

The experimental evidence described in the present paper provides convincing evidence that a marked delay and partial suppression of the rise in potassium conductance during activity are responsible for the increase in negative after-potential caused by DDT. The sodium inactivation process which occurs during the latter half of the spike is unlikely to be inhibited by DDT. This view strongly supports our previous hypothesis. It is also consistent with Welsh & Gordon's (1947) hypothesis in the sense that DDT exerts its effect at the surface membrane of the axon.

However, the present results, together with our observations on calcium action (Narahashi & Yamasaki, 1960), do not seem to fit with Welsh and Gordon's hypothesis, in that the action of DDT cannot be linked with calcium in the manner suggested by the hypothesis. The reasons for this supposition are as follows: (*a*) The resting potential, the magnitude and the maximum rates of rise and fall of the action potential, and the height of the negative after-potential, are all reduced by lowering the external calcium, whereas these changes are not caused by DDT. (*b*) The giant axon of the cockroach, even when treated with low-calcium solutions, neither gives rise to repetitive activity in response to a single stimulus nor discharges impulses spontaneously; this is in striking contrast with the results for lobster and crayfish axons, with which repetitive or spontaneous activity is observed when the external calcium is lowered (Adelman, 1956; Gordon & Welsh, 1948). (*c*) The repetitive response of the DDT-poisoned cockroach axon is not suppressed by raising the external [Ca]; this is also contrary to the results for crayfish axons, with which the repetitive response produced

by DDT is effectively suppressed by raising [Ca] (Gordon & Welsh, 1948). All these three arguments are incompatible with the idea that DDT competes with calcium for sites at the surface membrane.

The spike height during a repetitive response induced by DDT is increased by raising calcium. Since the proportion of the sodium-carrying system which is in an inactive state is decreased by raising calcium (Frankenhaeuser & Hodgkin, 1957), the improvement can be accounted for by a removal of inactivation.

Prolongation of the nerve action potential is known to be produced by treatment with certain drugs or ions such as veratrine alkaloids (Shanes, 1958), TEA (Burke, Katz & Machne, 1953; Tasaki & Hagiwara, 1957), high concentrations of glycerin (Mueller, 1958), and cobalt and nickel ions (Matsumoto, 1959; Sasaki, 1959). Among these, TEA was demonstrated to depress delayed rectification in the nerve cells of *Onchidium* (Hagiwara & Saito, 1959). It is apparent that TEA resembles DDT in the sense that the rectifying property of the membrane is suppressed. On the other hand, the veratrine-induced negative after-potential is different from that induced by DDT in that the former declines with a single exponential term (Shanes, Grundfest & Freygang, 1953), and may involve an increase in sodium conductance (Shanes, 1958).

It is interesting to note that the DDT-poisoned axon, when treated with potassium-free solution, gives rise to action potentials which resemble those of mammalian cardiac muscle fibres. The behaviour of the membrane potential under these conditions will be further described elsewhere.

SUMMARY

1. After treating a cockroach giant axon with DDT, the positive phase following the spike decreased and finally disappeared, and the negative after-potential increased until it attained a maximum, although the spike phase underwent little change. There was a brief period during which a repetitive response could be induced by a single shock. Thereafter, however, the rate of fall of the spike potential slowed considerably, and the negative after-potential finally began to decrease. Conduction block was never observed, and the resting potential underwent little change throughout.

2. Immediately after the repetitive period, damped oscillations were often seen on the declining phase of the increased negative after-potential.

3. In normal axons the time course of the decay of the negative after-potential following a single impulse could be expressed as a single exponential term. After treatment with DDT, however, the decay was initially slower than normal, and terminated in a faster phase.

4. After a train of impulses in a DDT-poisoned axon, the declining phase terminated in a very slow component which was less marked than in a normal axon, and there was little or no sign of the terminal fast phase seen after a single impulse.

5. Unlike normal axons, the negative after-potentials in the DDT-poisoned axon did not add linearly during a train of impulses.

6. Delayed rectification, which was always observed in the normal axons when cathodal current was applied, was found to be further delayed and partly suppressed in the DDT-poisoned axons. On cessation of cathodal current the electrotonic potential decayed more slowly in the DDT-poisoned axons than in the normal ones.

7. A rise in external potassium concentration reduced the DDT-induced negative after-potential. In potassium-free solutions it was greatly augmented and a plateau appeared.

8. A rise in external calcium concentration caused a further increase in the DDT-induced negative after-potential.

9. It is concluded that the increase in negative after-potential caused by DDT is not caused by a rise in potassium accumulation near the nerve membrane, but by a delay and suppression of the rise in membrane potassium conductance which accelerates the falling phase of the action potential.

We are indebted to Dr S. Hagiwara and Dr A. M. Shanes for criticisms during the course of this work. Thanks are also due to Miss S. Hamada for technical assistance.

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THE ACTION OF ACETYLCHOLINE ON CONDUCTION IN MAMMALIAN NON-MYELINATED FIBRES AND ITS PREVENTION BY AN ANTICHOLINESTERASE

By CHRISTINE J. ARMETT* and J. M. RITCHIE

From the Department of Pharmacology, Albert Einstein College of Medicine, Yeshiva University, New York 61, *N.Y., U.S.A.*

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Although acetylcholine excites nerve *endings* (Brown & Gray, 1958; Douglas & Gray, 1953; Zotterman, 1953; Gray & Diamond, 1957; Diamond, 1959; Douglas & Ritchie, 1960), there is a lot of experimental evidence which suggests that it does not act on the main part of the fibre running in the nerve trunk. For example, it does not initiate impulses when injected close-arterially into mammalian nerve trunks or when applied to amphibian single nerve fibres (Jarrett, 1956), it does not depolarize the fibre or block conduction (Lorente de Nó, 1944; Straub, 1955) and it does not alter the membrane conductance of squid giant axons (Hodgkin, 1947). However, in a recent series of experiments where acetylcholine was injected close-arterially into the cat's skin to study its excitatory effect on the endings of sensory non-myelinated fibres of mammalian cutaneous nerves (Douglas & Ritchie, 1960), a striking change was often observed in the shape of an antidromic C potential recorded from the nerve near the point where it left the skin. The type of change observed suggested that the after-potential had been altered. The affected region of the nerve was close to the skin, and it was observed that often the close-arterial injection not only reached the skin, but also perfused the nerve. It thus seemed likely that externally applied acetylcholine *could* alter the electrophysiological properties of mammalian C fibres, but with the techniques then used no definite conclusions could be reached. The present experiments use a technique more suited to this particular problem and deal with the action of acetylcholine on non-myelinated fibres in isolated mammalian nerves.

METHODS

Adult rabbits were anaesthetized with urethane (1·6 g/kg) given into a marginal ear vein as a 25 % (w/v) solution. A length of about 60–70 mm of a cervical vagus nerve was excised and then desheathed under the microscope (magnification × 25) by dissection with sewing needles sharpened to fine scalpels. In several experiments a similar length of the thoracic

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vagus near the level of the diaphragm was used; according to Evans & Murray (1954) this nerve contains hardly any myelinated fibres (less than 0·3 % compared with about 13 % in the cervical portion of the vagus). The desheathed nerve was then mounted on the sucrose-gap apparatus (Fig. 1) which was devised by Stämpfli (1954) and described in detail by Straub (1956, 1957); this apparatus had previously been used to study the C fibres in mammalian cervical sympathetic trunks (Ritchie & Straub, 1957). A single electrical shock delivered from an RF isolation unit (Schmitt, 1948) to minimize shock artifact, could be applied through any two of four indwelling platinum electrodes (E_1, E_2, E_3, and E_4); usually E_1 and E_2 were used with the cathode at E_1. Rectangular pulses of current of 0·05 msec duration were used to excite the myelinated fibres and pulses of 0·5 msec duration to excite the non-myelinated fibres. The potentials were recorded at the junction between the fluid

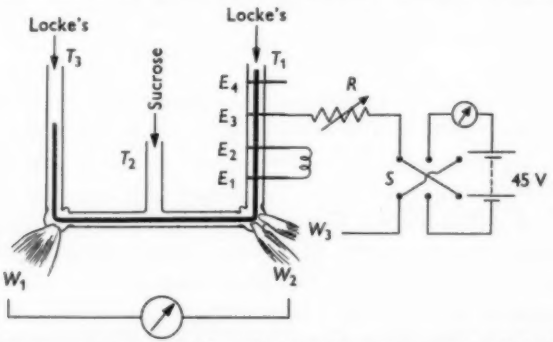

Fig. 1. The sucrose-gap apparatus showing the arrangement of electrodes for stimulating (E_1 and E_2), for recording (W_1 and W_2) and for applying external currents (W_3 and E_3). S is a reversing switch, and R a series resistor (0·1–100 MΩ). For detailed description, see text.

which flowed through the fine Perspex tube (T_1) and the isotonic sucrose solution in the middle Perspex tube (T_2); the distance from this junction to the stimulating cathode at E_1 was about 5 mm. These potentials were recorded relative to the potential which existed at the interface between the sucrose solution and the Locke's solution flowing through the Perspex tube (T_3) and which was assumed to remain constant throughout the experiment. The action potential and changes in the resting membrane potential were picked up through a pair of non-polarizable Ag–AgCl cotton wick electrodes (W_1, W_2), fed into a low-grid-current cathode-follower and then into a directly coupled amplifier. The potentials were displayed on a cathode-ray oscillograph and photographed on film. In some experiments small currents were passed through a Ag–AgCl cotton wick electrode (W_3) dipping into the pool between T_1 and T_2 and one of the indwelling platinum electrodes (E_3 or E_4). This current could be varied by means of a series resistor (R) and could hyperpolarize or depolarize the fibre at the point of recording by extra-polar spread, depending on the setting of a reversing switch (S). The rate of flow of the perfusion fluid was measured on a small flowmeter: it was usually about 0·5–1 ml./min. The temperature of the perfusion fluid near the recording sucrose–Locke's solution interface was measured by means of a fine thermocouple: it was usually about 30–35° C. The perfusion fluid in T_1 could quickly be changed by means of a tap (not shown in the diagram).

The composition of the Locke's solution used was (mM): NaCl, 154; KCl 5·5; $CaCl_2$, 2·2; dextrose, 5·0. It was buffered in a few early experiments by adding $NaHCO_3$ (final concentration, 1·9 mM) and bubbling into it a 5 % CO_2, 95 % O_2 mixture; but in most experiments it was buffered by adding a sodium phosphate buffer (1 or 2 mM) to bring the pH to about 7·0.

This latter solution was equilibrated with oxygen. The acetylcholine solutions used in the experiments were always freshly prepared from new phials of acetylcholine chloride (Merck) usually within a few minutes of testing its effect.

RESULTS

The vagal C potentials

The typical vagal compound action potential obtained with the present technique is illustrated in Fig. 2*a*. It consisted of two main elevations. The smaller was usually merged with the stimulus artifact, and was derived from the fast-conducting myelinated B fibres; the main component, which was usually between 5 and 20 mV in amplitude, was the C potential derived from the non-myelinated fibres. In this and in all subsequent records of the compound action potential the record of the combined B and C potentials obtained by using a strong shock was superimposed on a

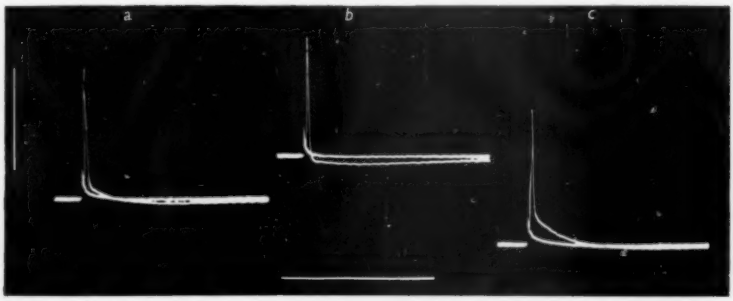

Fig. 2. The compound vagal action potential of the rabbit's cervical vagus nerve. The B potential is lost in the stimulus artifact. The main elevation is the C potential of the non-myelinated fibres. The records were taken: (*a*) in the normal nerve; (*b*) during the passage of a depolarizing current of 15 μA between W_3 and E_3 (Fig. 1); (*c*) during the passage of a hyperpolarizing current of 15 μA. The vertical bar represents 5 mV and the horizontal bar 250 msec. The temperature of the nerve was 35·2° C.

record of the B potential only, which was obtained immediately beforehand and which thus provided a base line for the C potential. The C spike was followed by a large after-positivity which in a series of 20 *fresh* nerves in phosphate-buffered Lock's solution varied from 0 to 7·5 % of the spike height (except in one nerve where it was 15·5 %), the average being $4{\cdot}0 \pm 0{\cdot}8$ % (S.E.) of the spike. No preparation ever gave the large positive after-potentials, up to 30 % of the spike height, which Gasser (1950) postulated in his reconstruction of the C potential of the saphenous nerve. The after-positivity tended to become more pronounced in preparations which had been mounted for some time. It was enhanced by

depolarizing the fibre by external currents (Fig. 2*b*) and abolished by hyperpolarizing the fibre (Fig. 2*c*). These latter observations suggest that the origin of this after-positivity is the same as that suggested by Frankenhaeuser & Hodgkin (1956) for the positive phase of the spike in the squid axon. They point out that if the resting membrane potential is below the potassium equilibrium potential (which it usually is) the rise in the membrane permeability to potassium ions persisting immediately after the spike (Hodgkin & Huxley, 1952) will give rise to a transitory hyperpolarization.

The action of acetylcholine on the vagal C fibres

The effect of acetylcholine on the resting potential. When the Locke's solution in T_1 bathing the nerve was switched to a solution containing acetylcholine in a concentration of 3×10^{-4} (w/v), i.e. a concentration of about 1·6 mM, there was a rapid fall in the membrane potential (Fig. 3) after an initial delay of about 20 sec, which represented the time taken to clear the dead space of the switching apparatus. On being returned to

Fig. 3. The effect of acetylcholine on the resting membrane potential of the rabbit's cervical vagus nerve. At the first marker the perfusing fluid was changed from Locke's solution to one containing 3×10^{-4} acetylcholine. At the second marker perfusion with the original Locke's solution was resumed. An upward deflexion indicates depolarization. The vertical bar represents 5 mV and the horizontal bar 3 min. The temperature of the nerve was 35·1° C.

Locke's solution the membrane repolarized, although recovery was relatively slow and was often not complete. The depolarization was usually measured 3 min after switching to Locke's solution. It nearly always occurred (one exception was observed in sixteen experiments, when there was a slight hyperpolarization which may have been due to amplifier drift). In experiments on sixteen different nerves the average depolarization after 3 min was $1{\cdot}23 \pm 0{\cdot}19$ mV. However, the maximum depolarization sometimes occurred earlier than this, as in the experiment of Fig. 7.

With a smaller concentration of 10^{-4}, little or no change in the resting potential was observed. In seven of the experiments described above, where a concentration of 3×10^{-4} was used, a concentration of 6×10^{-4}

was also tested. It was found to produce, on the average, about 140% of the depolarization that the smaller, standard, concentration produced. In three experiments a concentration of 10^{-3} was tested, producing an even greater depolarization, the average being about 180% of that obtained with a concentration of 3×10^{-4}.

Because of the small degree of short-circuiting of the fibres with the sucrose-gap technique these *measured* membrane potential changes are assumed to be close to the *actual* membrane potential changes. The absolute value of the resting potential could not, unfortunately, be determined, for with the particular sucrose-gap (T_2 in Fig. 2) which was used in the present experiments the nerve preparation only partly filled the lumen. This meant that when the preparation was perfused with isotonic potassium chloride solution instead of Locke's solution, in an attempt to measure the resting potential of the fibres, a large part of the recorded depolarization must have been caused by the more rapid diffusion of potassium than of sodium into the sucrose solution. Indeed, when the nerve preparation was replaced by a piece of thread of the same or smaller diameter, depolarizations of over 20 mV were obtained by changing the perfusion fluid from an isotonic sodium chloride solution to an isotonic potassium chloride solution; similar *hyper*polarizations were obtained on changing the perfusion fluid from an isotonic sodium chloride solution to an isotonic lithium chloride solution, as would be expected from the order of the mobilities of the lithium, sodium and potassium ions. The depolarization with acetylcholine in the nerve experiments, however, reflected an effect of the drug on the nerve membrane and was not a diffusion potential, for the diffusion effect with acetylcholine (and with choline) was found to produce a hyperpolarization, as would be expected with ions less mobile than the sodium ion. The small concentrations of acetylcholine used in the present nerve experiments did not in fact produce any appreciable diffusion potential.

The effect of acetylcholine on spike height. The depolarization of the C fibres by acetylcholine was accompanied by a fall in spike height. This is illustrated in Fig. 4, which shows the results obtained in two typical experiments. The upper left-hand record *a* is the control record taken shortly before one of the nerves was perfused with a concentration of 3×10^{-4} acetylcholine. The middle record (*b*) was taken 3 min after the change over had taken place. It is clear that not only is there a depolarization of about 2·03 mV (indicated by the shift in the level of the record) but that there is also a fall of about 2·55 mV (i.e. 18%) in spike height. The lower 3 records show the effect of a 3 min exposure to a lower concentration, 10^{-4}, of acetylcholine (*e*) and the subsequent recovery on resuming perfusion by Locke's solution (*f*). In observations made repeatedly on

more than thirty different nerves, this reduction in spike height was *always* obtained on exposure of the preparation to an acetylcholine concentration of 3×10^{-4}; recovery was usually complete within 6 min of the nerve being returned to Locke's solution. In eighteen nerves where the acetylcholine was tested on the fresh preparation in phosphate-buffered Locke's solution, the average fall after 3 min exposure to acetylcholine was 2·79 mV, with a standard deviation of 1·15 mV (S.E. $\pm$ 0·27); this corresponded to an average fall of 32·2 % in the spike height, with a

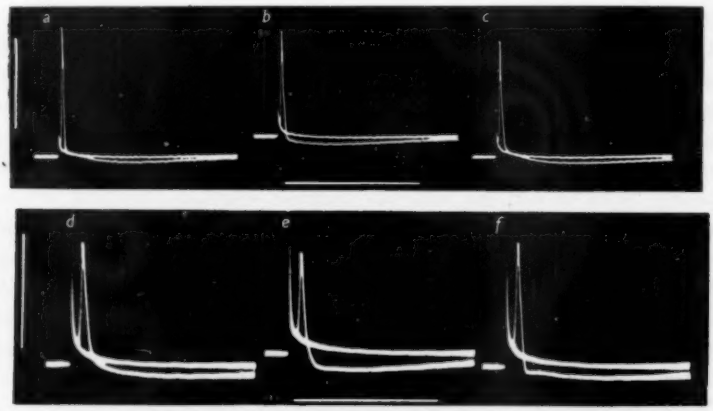

Fig. 4. The effect of acetylcholine on the C potential of the rabbit's cervical vagus nerve. The upper three records (*a*, *b* and *c*) were taken from one preparation and the lower three (*d*, *e* and *f*) from another. Record *a* was taken just before and record *b* 3 min after exposure of the nerve to 3×10^{-4} acetylcholine; record *c* was taken 15 sec after *b* with the preparation still in the acetylcholine solution, but hyperpolarized by passing 7·7 μA between W_3 and E_3 (Fig. 1). Record *d* was taken just before, and record *e* 3 min after exposure to 10^{-4} acetylcholine. Record *f* was taken 6 min after the drug had been removed. In this and all subsequent figures (except Fig. 6 and 11) the vertical displacement of the records reflects the changes in resting membrane potential. In the upper set of records the vertical bar represents 10 mV and the horizontal bar 250 msec; the corresponding bars in the lower set represent 5 mV and 100 msec, respectively. The temperature of the nerve in the upper set was 35·3° C; the temperature of that in the lower set was not measured.

standard deviation of 7·5 % (S.E. $\pm$ 1·8 %). Higher doses produced larger falls; this is illustrated in Fig. 5 which shows the results of one experiment on a particularly sensitive preparation, where the response to four different concentrations of acetylcholine was tested. In seven experiments where a concentration of 6×10^{-4} was used, the reduction in size of the action potential was on average 45 % greater than the reduction with the standard concentration of 3×10^{-4} obtained about 10 min beforehand; in five

similar experiments where a concentration of 10^{-3} was used the corresponding reduction was 90% greater than with 3×10^{-4}. In some other experiments where only a concentration of 10^{-3} was tested complete block occurred. A concentration of 10^{-4} was usually ineffective, but as has been shown in Fig. 4*e*, it could on occasion reduce the spike.

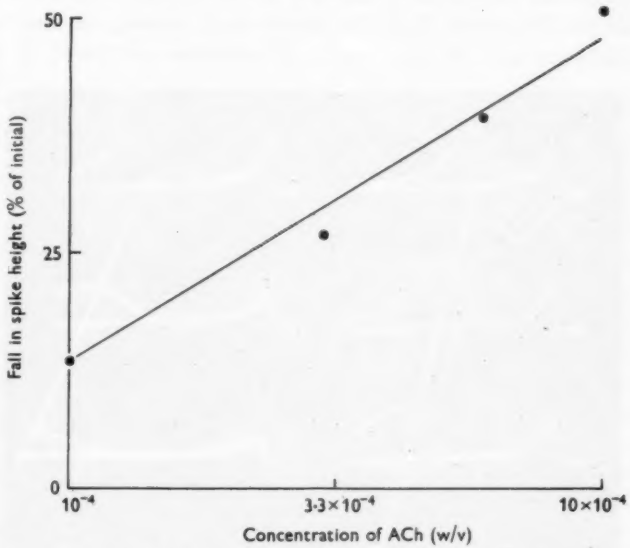

Fig. 5. The relationship between the concentration of acetylcholine in the perfusion fluid and the reduction in the height of the C potential of the rabbit's vagus nerve. Same nerve as in Fig. 4 (lower set). Note that the concentration scale is logarithmic.

The effect of acetylcholine on conduction velocity. Even in records of the C potential such as those in Fig. 4, which were taken on a slow time base, it could be seen that acetylcholine delayed the spike. This effect is seen particularly clearly with a faster time base, as in the experiment of Fig. 6. In this experiment each of the four indwelling platinum stimulating electrodes was used in turn as the stimulating cathode so that an accurate estimate of the conduction velocity could be obtained by the graphical method illustrated in the figure. The peak of the C potential in this preparation was found to be conducted at 0·99 m/sec at 35° C. Ten minutes after the preparation had been exposed to a concentration of 6×10^{-4} acetylcholine a similar determination of conduction velocity showed the peak to be conducted at only 0·57 m/sec, i.e. a slowing of over 40% had occurred.

Since bundles of nerve fibres rather than single axons were used in the

present experiments, this slowing in conduction must have contributed to the fall in spike height, for the more time that is allowed for temporal dispersion to occur, the smaller the action potential of the whole nerve becomes (Ritchie & Straub, 1956). A similar explanation accounts for the further observation, illustrated in Fig. 6, that the percentage fall in spike height was greater with long conduction distances than with short conduction distances. Thus with E_1 as the stimulating cathode the smallest conduction distance was obtained, and acetylcholine was found to produce

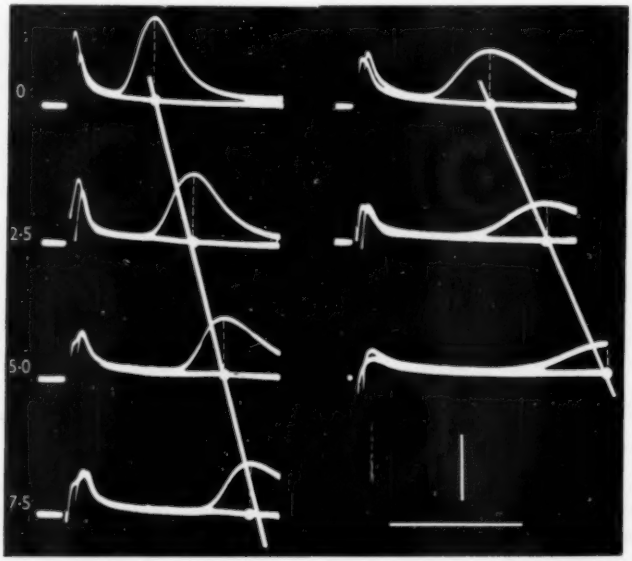

Fig. 6. The effect of acetylcholine on the conduction velocity of the non-myelinated fibres of the rabbit's vagus nerve. The four left-hand records were taken in Locke's solution. They were obtained by using E_1, E_2, E_3 and E_4 (Fig. 1) in turn as the stimulating cathode. The numbers at the side of each trace indicate the distances in millimetres of the stimulating cathode from E_1. The vertical positioning of each trace in the composite figure is proportional to this distance. The three right-hand traces are the corresponding records (using E_1, E_2 and E_3) obtained 10 min after the preparation was exposed to a concentration of 6×10^{-4} acetylcholine. The vertical bar represents 5 mV and the horizontal bar 10 msec. The temperature of the preparation was 34° C.

a 34·9 % fall in spike height (top pair of records), whereas with E_2 and E_3 as the stimulating cathodes the corresponding effects were 45·5 and 45·9 %, respectively: with E_4 as the stimulating cathode the reduction in spike height was 65·2 %.

The effect of acetylcholine on the positive after-potential. It can be seen in Fig. 4 that acetylcholine, in addition to depolarizing the fibre and

reducing the spike, has changed the form of the positive after-potential. The main change, which nearly always occurred when the after-potential could be resolved in the records, was that its peak occurred earlier; the acetylcholine also often caused the positive after-potential to end slightly earlier and to increase its amplitude at its peak. These changes were particularly pronounced in early experiments with the bicarbonate-buffered Locke's solution, but this may have been due to chance rather than to an effect of the bicarbonate ion.

The effect of hyperpolarizing currents on the action of acetylcholine

Depolarization of the fibre with external currents reproduces many of the effects of perfusion with acetylcholine. This can be seen by comparing the C potential of the depolarized fibres in Locke's solution shown in Fig. 2*b* with the C potential obtained when the same preparation was exposed to a concentration of 3×10^{-4} acetylcholine shown in Fig. 4*b*. The records are remarkably similar to one another; in both the spike height is reduced and the after-positivity enhanced. It therefore seemed worth while to find out whether or not the action of acetylcholine could be reversed by hyperpolarizing the fibres with externally applied currents. The results of one such experiment are illustrated in Fig. 4*c*, where it can be seen that the after-potential changes produced in the presence of acetylcholine have been reversed by means of a small hyperpolarizing current. However, the spike height, though increased by this procedure, was never returned to its original level. It seems therefore that the effects of acetylcholine in reducing spike height may not be entirely caused by the acetylcholine depolarization.

The time course of the action of acetylcholine

The resting and action potentials. In most experiments the nerve was stimulated infrequently, once every 3 min. The long interval between stimuli was chosen because, as Gasser (1950) has emphasized, previous activity alters the shape of the spike. Any such alteration as a result of the stimulation technique would have confused the interpretation of the action of acetylcholine. In a few experiments, however, the stimulation frequency was increased to 4 shocks/min to allow the time courses of the effects of acetylcholine on spike height and on resting potential to be obtained. The first portion of a series of records taken in such an experiment is shown in Fig. 7. The records show that the reduction in spike height and the depolarization occur *pari passu*. This is seen particularly clearly when the time courses of the depolarization and of the reduction in spike height are plotted together, as in Fig. 8. In this experiment there was actually some recovery in the membrane potential even when the

fibre was still exposed to the acetylcholine; this recovery in membrane potential was accompanied by a partial recovery in size of the spike.

In experiments such as that illustrated in Fig. 8 the size of the effect on the resting potential recorded 3 min after exposure to the drug was smaller than the maximal effect occurring about 1 min after the exposure. This early maximum was usually followed by a more slowly developing effect. Thus, in most of the experiments where records were taken only

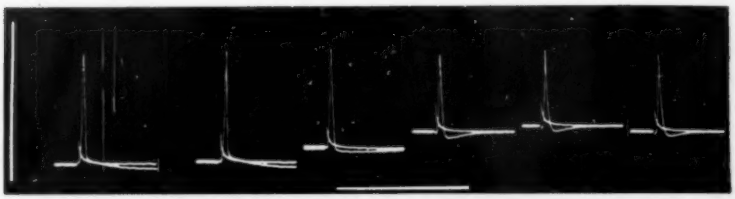

Fig. 7. Records showing the early time course of the action of acetylcholine on the non-myelinated fibres of the rabbit's vagus nerve. The records were taken every 15 sec. The left-hand record is the control and the switch to perfusion with 3×10^{-4} (w/v) acetylcholine occurred immediately after this record was taken. The vertical bar represents 10 mV and the horizontal bar 250 msec. The temperature of the nerve was 34·8° C.

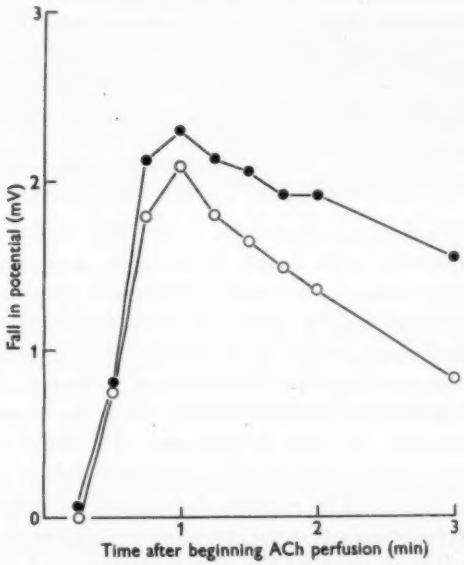

Fig. 8. The time course of the action of acetylcholine (3×10^{-4}) on the resting membrane potential (open circles) and on the amplitude of the C potential of the rabbit's vagus nerve (solid circles). The temperature of the nerve was 34·8° C.

every 3 min, the depolarization tended to increase slowly in successive records. This was parallelled by a continued increase in the effect of acetylcholine in reducing spike height. This is illustrated by the experiment of Fig. 9, which shows that in a preparation where a 3 min exposure to 3×10^{-4} acetylcholine produced a typical 32·6 % fall in spike height, longer exposures of 6 and 9 min produced falls of 50·0 and 58·9 % of spike height, respectively.

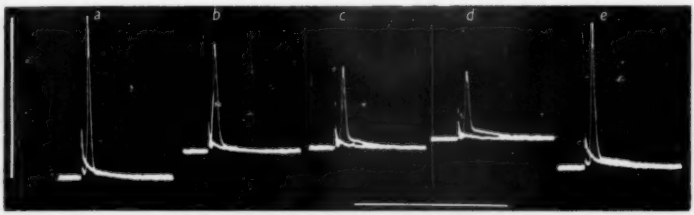

Fig. 9. The effect of prolonged exposure to acetylcholine on the non-myelinated fibres of the rabbit's cervical vagus nerve. Record *a* was taken before, and records *b*, *c* and *d* were taken 3, 6 and 9 min respectively after perfusion with 3×10^{-4} acetylcholine was begun. Record *e* was taken 12 min after the preparation was returned to Locke's solution. The vertical bar represents 5 mV and the horizontal bar 250 msec. The temperature of the nerve was 34·2° C.

The positive after-potential. Figure 7 illustrates a further typical finding in these experiments. The positive after-potential was particularly pronounced within the first minute after switching to perfusion by an acetylcholine solution, becoming larger in amplitude and more transitory than the type of after-potential which was recorded after exposure of 3 min or more (as in Fig. 4*b*).

Conduction velocity. The time course of the effect of acetylcholine on conduction velocity was determined by observing the progressive changes in the latency of C potential after exposure of the fibres to acetylcholine. As can be seen in Fig. 10 (solid circles) the latency changes occurred in two phases: there was first a slight reduction and then an increase. The effect of acetylcholine on spike height (open circles) also occurred in two phases. These correspond with the early and late phases previously illustrated in Figs. 8 and 9, which also indicate that there is an early and a late phase of depolarization.

A comparison of Figs. 8 and 9 and 10 makes it clear that the two phases of reduction in spike height, of depolarization and of latency changes correspond in time with one another. There is a rapid phase which reaches maximum about 1 min after switching to perfusion with acetylcholine (i.e. within $\frac{1}{2}$ min of the drug reaching the preparation) and a much slower phase which requires more than 5–6 min to reach its maximum.

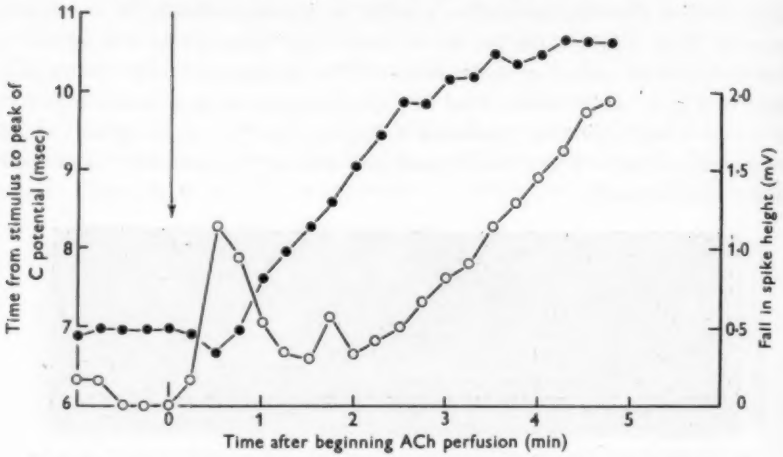

Fig. 10. The time course of the effect of acetylcholine on the conduction velocity of the non-myelinated fibres in a rabbit's cervical vagus nerve. The abscissae are the times after first exposing the preparation to a concentration of 6×10^{-4} acetylcholine, and the ordinate is the latency of the peak of the C potential (solid circles) and the reduction in spike height (open circles). The temperature of the nerve was 34° C.

The effect of acetylcholine on the myelinated B fibres

The action of acetylcholine on the myelinated vagal B fibres was not studied systematically because our main interest lay in its action on the C fibres. But it was often observed that the concentrations of acetylcholine which reduced the C potential also reduced the B elevation of the compound action potential. This reduction might have been caused by a direct action of acetylcholine on B fibres; or it might have occurred because the partially depolarized C fibres presented a smaller short-circuiting resistance to the B fibres than did the C fibres in Locke's solution, thus reducing the B potential. This latter possibility has not yet been tested directly. There is no doubt, however, that the reduction of the C potential is a direct effect of acetylcholine and is not caused by an increased short-circuiting of affected B fibres, for typical effects of acetylcholine were obtained in several experiments on the lower portion of the thoracic vagus nerve at the level of the diaphragm, which contained hardly any myelinated fibres (Evans & Murray, 1954) and which was found in the present experiments to have an insignificant B potential.

The effect of eserine on the action of acetylcholine

The action of acetylcholine in depolarizing nerve fibres seems in some ways analogous to its action in depolarizing various junctional tissues,

such as the motor end-plate and the ganglionic synapse. It was expected, therefore, that anticholinesterases would potentiate the action of this drug on nerve fibres, as it does elsewhere, by preventing the tissue break-down of acetylcholine. We were therefore surprised to find that in the presence of eserine salicylate ($1–10 \times 10^{-5}$, w/v), acetylcholine had a *smaller* effect on the spike height (Fig. 11). The effect of eserine in blocking the action of acetylcholine may be the same as that reported by Dettbarn (1959) who found that it blocked the depolarizing action of various lipid-soluble quaternary ammonium ions on the myelinated fibres of the frog.

Whereas eserine in weak concentrations (10^{-5} or less) had no action by itself on spike height, eserine in stronger concentrations ($3 \times 10^{-5}–10^{-4}$) always reduced the spike (by an average of 10·6 % in 3 min in four experiments and by greater amounts at longer times); there was, however, no detectable depolarization with these concentrations. An even stronger concentration (10^{-3}) produced a complete block in conduction. This block was partially reversed by applying a hyperpolarizing current, as Wright (1956) had previously noted. Concentrations of eserine as low as this (0·08–0·2 mM to reduce spike height, and 2 mM to produce block) have not previously been reported to be effective on myelinated fibres (Langley & Dickinson, 1890; Lorente de Nó, 1944; Straub, 1955; Wright, 1956) or on squid nerve fibres (Bullock, Nachmansohn & Rothenberg, 1946).

DISCUSSION

The main conclusion to be drawn from these experiments is that acetylcholine does have a definite action on certain peripheral nerve axons; it depolarizes the axons of vagal non-myelinated fibres, it reduces their spike height, it slows conduction in them and it causes the positive after-potential in these fibres to become more rapid in onset and often to become larger in amplitude. The changes in the after-potential would, of course, account for the original observation of the effect of acetylcholine on nerve fibres made by Douglas & Ritchie (1960). In their experiments, electrical shocks were applied to the cat's saphenous nerve high in the thigh and the evoked antidromic action potentials were recorded *diphasically* by a pair of electrodes on a small nerve branch just before it entered the skin near the knee. They noted that the conventional negative followed by positive deflexions of the diphasic records were succeeded by a long-lasting apparent negativity. This could have been due to the appearance of a negative after-potential at the recording electrode nearer the stimulating pair. However, another possibility is that it was due to the enhancement of a positive after-potential at the other, more distant, recording electrode; either an increase in its peak height or a more rapid onset of the after-potential would be sufficient. Since

acetylcholine has now been shown to have *both* these effects on the positive after-potential and since the more distant recording electrode was the more likely to be reached by the close arterial injection (being nearer the skin) it seems that the second possibility is the one which most likely accounts for the phenomenon observed by Douglas & Ritchie (1960).

The question which immediately arises is why no depolarization, reduction in spike height, or blockage of nerve conduction was noted previously. Clearly, it has not been due to oversight, for many studies have been made to see whether or not such effects occur (e.g. Lorente de Nó, 1944; Straub, 1955) particularly because of the important role which acetylcholine is sometimes assumed to play in nervous conduction (see Nachmansohn, 1959). One possible explanation for the success of the present experiments in demonstrating an action of acetylcholine is that surface phenomena (such as membrane potential changes) are better seen in non-myelinated fibres because they have a particularly large surface:

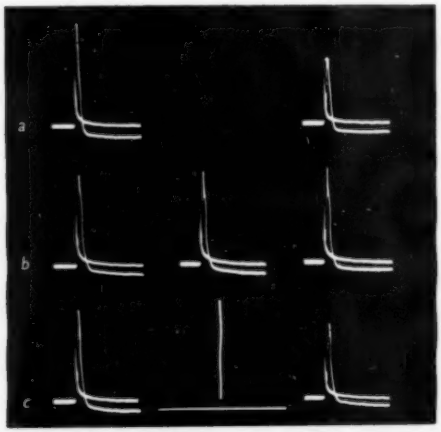

Fig. 11. Records of the C potential of the rabbit's cervical vagus nerve taken to test the effect of eserine on the action of acetylcholine on the C fibres. The records on the left-hand side were all taken with the nerve in Locke's solution; those on the right-hand side were all taken after 6 min exposure to a concentration of 3×10^{-4} acetylcholine. Each set of records has been brought to the same base line. The top pair of records, *a*, shows the effect of acetylcholine on the untreated nerve. Between taking the records on the left-hand side and the middle records in *b*, eserine (10^{-5}) was added to the perfusion fluid and was still present when the effect of acetylcholine was subsequently tested (right-hand records). The pair of records in *c* was taken 33 min after the last of the records in *b* during which time the fibre was bathed in Locke's solution (with no eserine in it). Note the partial recovery in the action of acetylcholine. The vertical bar represents 5 mV and the horizontal bar 250 msec. The temperature of the nerve was 34·2° C.

volume ratio. This ratio is much larger in the small non-myelinated vagal fibres than in the larger fibres used previously, such as the myelinated fibres of amphibia or the squid fibres. It seems from these experiments, and from others now being made on the effects of neurotropic drugs on nerve fibres (P. Greengard & J. M. Ritchie, unpublished), that these small fibres may be intermediate, as far as their pharmacological reactions are concerned, between the more commonly studied peripheral axons which tend to be relatively insensitive to applied drugs and the nerve-cell bodies with their dendrites.

The present experiments were carried out on nerve bundles rather than on single fibres. It is therefore difficult to determine whether the effect of acetylcholine on the membrane of an individual fibre is really graded, getting greater as the concentration of the drug is increased, or whether the observed decrease in the C potential is caused by an increasing *number* of fibres becoming *completely* blocked. Furthermore, because temporal dispersion occurs under the experimental conditions, a reduction in the C potential could have been caused without any fall in spike height of the individual fibres either if the spike became shorter in duration or if the conduction velocity decreased. The depolarization by itself must have led to a decrease in the spike height of the individual fibres, for in addition to lowering the potential from which the spike started it must also have reduced the amount of overshoot by partially inactivating the sodium carrier mechanism (Hodgkin & Huxley, 1952). How much the observed decrease in the C potential of the whole nerve is due to this factor and how much it is due to the increased dispersion allowed by the decreased conduction velocity is not known at the moment.

Our experiments do not yet allow us to decide how acetylcholine acts, whether, for example it acts by causing a non-specific increase in membrane permeability to all ions as it does at the motor end-plate (Fatt & Katz, 1951) or whether, for example, it increases the membrane permeability to one specific ion such as the sodium ion. However, our experiments do indicate something about the type of receptor involved. Whatever this receptor is, it is blocked by the anticholinesterase eserine. This suggests that the receptor is similar to, though not necessarily the same as, cholinesterase itself: and that the reaction between acetylcholine and the receptor cannot occur in the presence of an anticholinesterase. Whatever the explanation, the experiments clearly demonstrate a paradoxical effect of an anticholinesterase, namely that it inhibits rather than enhances the effect of applied acetylcholine. This is not, however, the first time that such an effect has been noted, for Feldberg & Vartiainen (1935) observed that although eserine, when injected into the cat's superior cervical ganglion in small amounts (concentration in the perfusion fluid, 10^{-6}) enhanced

the excitant action of a subsequent injection of acetylcholine, a stronger concentration (10^{-4}) rendered the ganglion cell unresponsive to acetylcholine. This effect, which they attributed to a nicotine-like action of eserine, may well have been caused by an action of eserine similar to that described in the present paper. Furthermore Quilliam & Strong (1949) observed in isolated perfused hearts which had been exposed to DFP and which consequently were very sensitive to applied acetylcholine, that subsequent exposure to the anticholinesterases eserine or prostigmine decreased this sensitivity.

One possible explanation which would account for our results is that the effects of acetylcholine on the resting and action potentials depend on the action of some of the products of hydrolysis of acetylcholine. We have tried to test this possibility by examining the effects of perfusing the fibre with choline chloride, with sodium acetate and with solutions where the pH had been lowered. Both the choline and the more acid Locke's solutions reduced spike height, but the effects produced were rather small, the fall being only 10–20 % even when the preparation was perfused with a relatively large concentration of choline chloride (15 mM) or when the pH was changed by as much as 0·5 unit; sodium acetate (15 mM) had little or no effect on spike height. These changes in choline, acetate and hydrogen ion concentration are much larger than could be produced in the bulk of the solution or on the axoplasm generally; but the possibility that changes of this size occur locally in particular regions of the membrane cannot be excluded. These experiments therefore do not as yet shed much light on the mode of action of acetylcholine on the nerve axon.

It is tempting to speculate that the sensitivity of peripheral nerve endings to acetylcholine (Brown & Gray, 1948; Douglas & Gray, 1953; Douglas & Ritchie, 1960) reflects the sensitivity of non-myelinated axons shown in the present studies, for whether or not the fibres are myelinated their endings are non-myelinated. The nerve endings are more sensitive than the nerve axons by a factor of at least 10; the threshold response of the endings is obtained with a close-arterial *injection* of 10^{-5} acetylcholine, whereas the threshold response of the fibre is obtained with *perfusion* with a concentration of about 10^{-4} of acetylcholine; but this difference might possibly be explained on anatomical grounds. However, there is one important difference between the two preparations. Acetylcholine initiates conducted impulses at sensory nerve endings, but there is no evidence that it does so in axons from experiments where it was injected into normal nerve trunks (Diamond, 1959), or from the present perfusion experiments even when the amounts were big enough to lead eventually to complete block in conduction. Furthermore, the excitatory

action of acetylcholine at the endings is certainly not reduced by anticholinesterases (Brown & Gray, 1948). This is just the opposite to what occurs with the axon, where the action of acetylcholine has now been shown to be much reduced by the anticholinesterase eserine. It seems therefore that acetylcholine acts in quite different ways at the two sites, reacting with quite different types of receptor.

SUMMARY

1. The sucrose-gap technique has been used to record the compound action potentials of desheathed bundles of vagal fibres and to study the action of acetylcholine on the numerous non-myelinated fibres in this nerve.

2. The action potential of the C fibres is followed by a long-lasting after positivity whose peak amplitude is about 4·0 % of the spike height.

3. When the preparation is perfused with acetylcholine (3×10^{-4} w/v) the resting potential falls by more than 1 mV, the spike amplitude is reduced by about 30 %, the conduction velocity is slowed and the after-positivity is enhanced.

4. Larger effects are obtained with larger concentrations; threshold effects are observed with a lower concentration, 10^{-4}.

5. High concentrations of acetylcholine (10^{-3}) sometimes completely block conduction.

6. These changes are rapidly reversed when the acetylcholine is removed.

7. Eserine in high concentration has a similar effect on the spike to acetylcholine. In a concentration of from 3×10^{-5} to 10^{-4} it reduces spike height: a concentration of 10^{-3} completely blocks conduction.

8. The effects of acetylcholine are abolished or much reduced by the anticholinesterase eserine.

9. The action of eserine is reversible.

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SODIUM PERMEABILITY IN TOAD NERVE AND IN SQUID NERVE

BY B. FRANKENHAEUSER

From the Nobel Institute for Neurophysiology, Karolinska Institutet, Stockholm 60, *Sweden*

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The passive transport of sodium across the membrane of the squid giant nerve fibre has been successfully described in terms of the sodium conductance (g_{Na}) of the membrane (Hodgkin & Huxley, 1952*a–d*). The sodium conductance is defined as the ratio of the sodium current (I_{Na}) to the driving electromotive force for sodium ($V - V_{Na}$), i.e.

$$g_{Na} = \frac{I_{Na}}{V - V_{Na}}.$$

g_{Na} changes continuously and with finite speed when the membrane potential is changed. Since g_{Na} changes with finite speed it is clear that the instantaneous value of g_{Na} is the same immediately before and immediately after a sudden change in membrane potential. Thus g_{Na} is independent of the instantaneous value of membrane potential at which it is measured. In normal sea water g_{Na} was found experimentally to be independent of the instantaneous potential at which it was measured.

These relations do not hold, however, for the myelinated nerve fibre (Dodge & Frankenhaeuser, 1958, 1959). The $I_{Na} - V$ curve is not rectilinear at large values of V, but is clearly curved, showing a rectification such that g_{Na} would decrease at large values of V. Further the instantaneous value of g_{Na} is not independent of the instantaneous potential at which g_{Na} is measured. If the toad fibre voltage clamp data are described in terms of the sodium permeability (P_{Na}) of the membrane, then this ambiguity vanishes and P_{Na} is independent of the instantaneous value of V at which it is measured. P_{Na} is here defined by the constant field equation (Goldman, 1943; Hodgkin & Katz, 1949; Dodge & Frankenhaeuser, 1959).

P_{Na} (of the toad nerve) can be directly compared with g_{Na} (of the squid nerve) only under certain conditions. One such condition is when the membrane potential is zero ($E = 0$). Since in this case there is no net electromotive driving force, ion transport is due to diffusion, and therefore the sodium permeability is proportional to the inward flow of sodium

divided by the concentration difference of sodium on the two sides of the membrane ($[Na]_o - [Na]_i$). Thus

$$\frac{P^X_{Na}}{P^L_{Na}} = \frac{I^X_{Na}\,[Na]^L_o - [Na]^L_i}{I^L_{Na}\,[Na]^X_o - [Na]^X_i}. \quad (1)$$

Where X and L indicate results respectively from *Xenopus* fibres and *Loligo* fibres. Another condition where a comparison is possible is obtained when the constant field equation is modified in such a way that it agrees with the squid fibre results.

TABLE 1

	Xenopus Axon	E_{Na} (mV)	$[Na]_i$ (mM)	$[Na]_o - [Na]_i$ (mM)	h_0	$I^{(h_0)\,(E=0)}_{Na}$ (mA/cm²)	$I^{(h=1)\,(E=0)}_{Na}$ (mA/cm²)	$g^{(h_0)}_{Na}$ (m-mho/cm²)	$g^{(h=1)}_{Na}$ (m-mho/cm²)
A	7, 20° C	+60·0	10·4	104·1	0·95	−36·3	−38·2	—	—
B	8, 20° C	+62·0	9·6	104·9	0·57	−17·9	−31·4	—	—
C	9, 20° C	+56·2	12·1	102·4	0·89	−33·9	−38·1	—	—
D	10, 20° C	+50·0	15·5	99·0	0·77	−23·0	−29·9	—	—
E	11, 20° C	+46·0	18·2	96·3	0·59	−22·8	−38·6	—	—
F	12, 20° C	+50·2	15·3	99·2	0·66	−39·5	−59·8	—	—
G	mean 20° C	+54·1	13·5	101·0	0·738	−28·9	−39·3	—	—
	Squid								
H	6° C	+45·0	70·4	389·6	0·6	− 1·4	− 2·3	30·0	50·0
I	6° C	+50·0	57·5	402·5	0·6	− 1·8	− 2·9	35·0	58·4
J	6° C	+47·5	64·0	396·0	0·6	− 1·5	− 2·6	32·5	54·2
K	20° C	+49·7	64·0	396·0	0·6	− 2·8	− 4·6	55·9	93·2

The experimental data required for the first comparison are collected in Table 1. The values for the toad fibres at 20° C were obtained in the following way: the resting potential was assumed to be −70 mV. I_{Na} at $E = 0$ was measured on the experimental records. E_{Na} was calculated from the earlier measurements of V_{Na} (Frankenhaeuser, 1959). $[Na]_i$ was calculated by the equation $[Na]_i = [Na]_o \exp(-E_{Na}F/RT)$. The values of h_0 for these fibres have been given in Table 1 in Frankenhaeuser (1959). The values for the squid fibre data at 6° C were obtained on the basis of those given by Hodgkin & Huxley (1952*a*, *c*); g_{Na} = 30–35 m-mho/cm², resting potential = −56 mV, E_{Na} = +45 to + 50 mV, $h_0 = 0{\cdot}6$ and $[Na]_o$ = 460 mM. Lines H and I give the upper and lower extreme values, J gives the values for 6° C used for the calculation of the ratio of the permeability in the two fibres, and line K has been corrected to be appropriate for 20° C, by using a temperature dependence of g_{Na} of 4% per degree centigrade as found by Moore (1958).

The ratio of P_{Na} in the *Xenopus* fibre to P_{Na} in the *Loligo* fibre (Table 3) was thus found to be 75·5, or 40·5 when the effect of temperature is corrected for, for fibres at resting potential before test, or 59·3 and 33·6 respectively, for anodally conditioned fibres with $h = 1$. On this basis, the toad

fibre is clearly more sodium-permeable than the squid fibre. There is at present no way of deciding whether the difference depends on differences in the number of sites or in the efficiency of single sites. It also does not account for the difference in the general shape of the $I_{Na}-V$ curve.

A second approach is to modify the constant-field assumptions in such a way that the field equation can be applied both to squid and myelinated axons. A convenient method, suggested by Professor A. L. Hodgkin, is to assume that the electric field in the membrane is modified by the presence of fixed charges on one side of the membrane. It is assumed that the effect of a positive charge, adsorbed on the outside, will be to give a potential step E' at the outer boundary and a uniform field of magnitude $E'/$(thickness of membrane).

In the absence of such an adsorbed charge, the expression for I_{Na} (cf. Goldman, 1943; Hodgkin & Katz, 1949) is

$$I_{Na} = \frac{u_{Na}FE}{a}\frac{\beta_{Na}[Na]_i - \beta_{Na}[Na]_o \exp(-EF/RT)}{1-\exp(-EF/RT)}, \quad (2)$$

where I_{Na} is the outward sodium current and E is the potential difference between the inside and outside of the fibre; R, T and F have their usual significance; a is the membrane thickness; u_{Na} is the mobility of sodium ions in the membrane; and β_{Na} is the partition coefficient between the membrane and the aqueous solution.

Bearing in mind that the effect of the potential step E' at the outside of the membrane is to change the membrane sodium concentration at the outside from $\beta[Na]_o$ to $\beta[Na]_o \exp(-E'F/RT)$ and that the potential gradient in the membrane is $(E-E')/a$ instead of E/a, it follows that

$$I_{Na} = \frac{u_{Na}\beta_{Na}F}{a}(E-E')\frac{[Na]_i - [Na]_o \exp(-E'F/RT)\exp\{-(E-E')F/RT\}}{1-\exp\{-(E-E')F/RT\}}. \quad (3)$$

Defining P_{Na} as $u_{Na}\beta_{Na}RT/aF$ and writing E_{Na} for $RT/F \ln [Na]_o/[Na]_i$ we obtain

$$P_{Na} = \frac{I_{Na}}{E-E'}\frac{RT}{F^2}\frac{\exp(E'F/RT)}{[Na]_o}\frac{\exp\{(E-E')F/RT\}-1}{\exp\{(E-E_{Na})F/RT\}-1}. \quad (4)$$

By a similar argument it can be shown that fixed negative charges adsorbed on the inner edge of the membrane will give

$$P_{Na} = \frac{I_{Na}}{E-E'}\frac{RT}{F^2[Na]_o}\frac{\exp\{(E-E')F/RT\}-1}{\exp\{(E-E_{Na})F/RT\}-1}. \quad (5)$$

Equations (4) and (5) share the common feature that they indicate no rectification in the special case that $E' = E_{Na}$, since equation (4) is then

$$P_{Na} = \frac{I_{Na}}{E - E_{Na}} \frac{RT \exp (E_{Na} F/RT)}{F^2 [Na]_o} \tag{4.1}$$

and equation (5) is $$P_{Na} = \frac{I_{Na}}{E - E_{Na}} \frac{RT}{F^2 [Na]_o}. \tag{5.1}$$

Since sodium conductance is defined as

$$g_{Na} = \frac{I_{Na}}{E - E_{Na}},$$

it is clear that P_{Na} can be calculated from the squid fibre data of g_{Na} by either equation (4.1) or (5.1). There is no indication whether (4.1) or (5.1) should be used for the calculation or whether an equation giving an intermediate value would be more appropriate. The last alternative would correspond to a situation where the hypothetical charges are localized somewhere between the inside and outside edges of the membrane.

TABLE 2. P_{Na} of squid axon with the properties given in lines J and K in Table 1

	Temp. (° C)	From eqn. (4.1) (cm/sec)	From eqn. (5.1) (cm/sec)
h_0	6	$12{\cdot}7 \times 10^{-5}$	$1{\cdot}8 \times 10^{-5}$
$h = 1$	6	$21{\cdot}2 \times 10^{-5}$	$2{\cdot}9 \times 10^{-5}$
h_0	20	$23{\cdot}2 \times 10^{-5}$	$3{\cdot}2 \times 10^{-5}$
$h = 1$	20	$38{\cdot}7 \times 10^{-5}$	$5{\cdot}3 \times 10^{-5}$

TABLE 3. P^x_{Na}/P^f_{Na} calculated in different ways

		From eqn. (1)	From eqn. (4.1)	From eqn. (5.1)
A	h_0	75·5	25·2	177·8
B	$h = 1$	59·3	20·8	151·7
C	h_0	40·5	13·8	100·0
D	$h = 1$	33·6	11·4	83·0

In A and B squid fibre data for 6° C; in C and D for 20° C. *Xenopus* data for 20° C.

A squid fibre membrane, with the characteristics listed in Table 1, would thus have the sodium permeabilities in Table 2 as calculated from equation (4.1) and (5.1). The available sodium permeability in the *Xenopus* fibre at 20° C was earlier (Frankenhaeuser, 1959, Table 1) found to be

$$P^{h_0}_{Na} = 3{\cdot}2 \times 10^{-3} \text{ cm/sec} \quad \text{and} \quad P^{h=1}_{Na} = 4{\cdot}4 \times 10^{-3} \text{ cm/sec}.$$

Table 3 gives the ratios of sodium permeability in the *Xenopus* fibre to that in the squid fibre as calculated in the three different ways. The *Xenopus* fibre data were obtained at 20° C throughout, the squid fibre data at 6° C. Lines A and B have not been corrected for the difference in

temperature. In lines C and D g_{Na} of the squid fibre was corrected for 20° C (Moore, 1958) and is therefore more likely to be correct. All the comparisons reveal that the toad fibre was more sodium-permeable than the squid fibre. This also holds true for the somewhat higher values of g_{Na} found by Moore (1959).

DISCUSSION

In the present study the sodium permeability of the toad myelinated fibre has been compared with that of the squid giant nerve fibre by using three equations based on different assumptions. Equation (1) was obtained on the basis that ion transport is due to diffusion only when membrane potential is zero. No specific assumptions are necessary for the membrane, the permeability may be restricted or increased by electric charges in addition to the partition coefficient (β in Hodgkin & Katz, 1949). The assumptions for equation (1) are thus very general and the membrane is treated as a unit. The limitations to this treatment are that it is applicable only to the special case that $E = 0$, and that it does not allow for the presence or absence of rectification.

Equations (4) and (5) are based, in addition to the assumptions for the constant-field equation (Goldman, 1943; Hodgkin & Katz, 1949), on a very specific assumption: a fixed charge at one of the boundaries of the membrane, resulting in a potential step (E') at this boundary. For (4) this charge is localized to the *outside* edge of the membrane and for (5) to the *inside* edge. The amount of rectification is determined by the value of E' but not by the localization of E'. The value of E' can therefore be experimentally determined from the rectification; in the toad fibre $E' = 0$, whereas in the squid fibre in normal $[Na]_o$ $E' = E_{Na}$. In calculation of P_{Na} from equations (4) and (5) it is clear that the effect of E' on the ionic currents is separated from the permeable channel. This is not the case in calculations with equation (1). The advantage of (4) and (5) over (1) is that the amount of rectification is accounted for, and that the equations are applicable to data obtained at any value of E. Further, the $I_{Na} - V$ relation in squid fibre in low $[Na]_o$ is not linear, and the currents cannot, in low $[Na]_o$, be described in terms of g_{Na}. Hodgkin & Huxley (1952*a*) used a special equation derived on the basis of the independence principle (equation 12 in their paper), to compare sodium currents in fibres in low and normal $[Na]_o$. Equation 12 does account for the rectification in the squid fibre in low $[Na]_o$. Hodgkin & Huxley point out that the independence principle also applies to the constant field equation. If E' is unaffected by sodium concentration, then equation 12 can be derived also from equation (4) and (5). These, therefore, account satisfactorily for the change in rectification when external sodium concentration is changed.

Both equation (4) and equation (5) thus account for the shape of the $I_{Na} - V$ curve of the squid fibre at large cathodal pulses. The absolute value of P_{Na} calculated from the $I_{Na} - V$ relation depends, however, on whether equation (4) or (5) is used. The assumptions on which the equations are based differ only in the assumed sites of the fixed charge, producing the potential step (E'): for (4) this is at the outside edge of the membrane, and for (5) at the inside edge. An intermediate localization of a step E' gives the same rectification, but the calculated value of P_{Na} would be intermediate between those calculated from (4) and (5). Equations (4) and (5) were chosen because these give the extreme values for any localization or any combination of E' as long as all of them have the same polarity. If several potential steps with different polarities are assumed, then a comparison between the permeabilities is impossible even when the net potential step is known, as long as both localization and magnitude of the individual steps are unknown.

In the formal treatment of equations (4) and (5) the field within the membrane was modified with a potential step, E', in order to modify the 'constant-field' equation to fit the squid fibre data. The effect of E' is to change the sodium concentration either at the outside edge or at the inside edge of the permeable site, and in the special case with no rectification (i.e. the squid fibre in normal $[Na]_o$), $E' = E_{Na}$, and sodium concentration is the same at the two edges of the permeable site. It is clear that some other treatment, which would have a corresponding effect on concentrations, would be equally consistent with the experimental data.

There are some possibilities of envisaging the mechanisms of the potential step, E', which seem so simple and straightforward that they are worth mentioning, in spite of the fact that they are entirely speculative:

(*a*) Fixed positive charges at the outside surface of the membrane would affect the ionic concentration at the outside edge of the permeable channel in a manner equivalent to the effect of E' in equation (4).

(*b*) Fixed negative charges on the inside surface would correspondingly be equivalent to E' in equation (5).

(*c*) Fixed dipole charges in the permeable site, with the positive charge outwards, would be equivalent to E' in an equation giving an intermediate value for P_{Na}.

In this connexion it may be pointed out that in the squid fibre potassium currents are described satisfactorily in terms of potassium conductance (g_K). E_{Na} is positive while E_K is negative. E' in the potassium channel ought therefore to have the opposite sign to E' in the sodium channel. This could be interpreted to indicate that the sodium- and potassium-

permeable sites in the squid fibre are separated from each other, and that if alternative (*a*) or (*b*) (above) is corrected then the surface charge would be non-homogeneous, while if (*c*) is correct then the dipoles would be turned in opposite directions in the two sites. Conclusions of this kind cannot yet be drawn from the voltage clamp data on *Xenopus* fibres, because here $E' = 0$ for the sodium channel, and the potassium-carrying mechanism has not yet been fully analysed.

SUMMARY

1. The sodium transport mechanism in axons from *Loligo* has previously been described in terms of sodium conductance (g_{Na}), but in axons from *Xenopus* in terms of sodium permeability (P_{Na}) as defined by the constant field equation. The sodium permeability of the two nerves was compared on the basis of certain assumptions.

2. The permeabilities were compared at zero membrane potential (eqn. 1) where sodium transport is due to diffusion only: (*a*) P_{Na} of the toad fibre was found to be about 30–40 times larger at 20° C than P_{Na} of the squid fibre. (*b*) The comparison does not account for the difference in rectification of the two fibres.

3. The constant field equation was modified to fit the squid fibre data by assuming the presence of adsorbed fixed positive charges on the outside boundary of the membrane (eqns. 4 and 4.1), or of negative charges on the inside boundary of the membrane (eqns. 5 and 5.1). As a result: (*a*) P_{Na} of the toad fibre was found to be about 11–14 times larger at 20° C than P_{Na} of the squid fibre according to equation (4.1), and correspondingly 80–100 times larger according to equation (5.1). (*b*) The effect of the fixed charges is separated from the permeable channel with this treatment. (*c*) Both equation (4) and equation (5) account for the absence of rectification in the squid fibre.

4. All comparisons reveal that the toad fibre has a larger peak P_{Na} than the squid fibre.

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NITROGEN ELIMINATION FROM THE TISSUES DURING OXYGEN BREATHING AND ITS RELATIONSHIP TO THE FAT:MUSCLE RATIO AND THE LOCALIZATION OF BENDS

By G. LUNDIN

From the Laboratory of Aviation Medicine, Institute of Physiology, University of Lund, Sweden

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In a previous paper (Lundin, 1953) it has been shown that the nitrogen eliminated from the tissues during oxygen breathing seems to be released in an exponential way in three stages. First, there is a rapid phase with an elimination half-time of about 1·5 min, then a slower phase with a half-time of 12–13 min and lastly a slow phase with a half-time of about 110–200 min. On theoretical grounds it is probable that the first phase corresponds to the nitrogen from the highly vascular tissues, such as liver, brain, heart, intestines, etc., the middle phase to nitrogen mainly from the muscles, and the slowest to nitrogen mainly from fat. Probably the free intestinal nitrogen escapes with the third fraction.

The values obtained above for the rapid and slow phases were very much the same as those obtained by Lawrence, Jones, Berg, Henry & Ivy (1948). However, the muscular phase had a higher elimination rate than that found in their experiments. Jones (1951) claims that the values of Lawrence *et al.* accord with the theory that the muscles give rise to the N_2 bubbles appearing during decompression, which are the cause of the group of symptoms called decompression sickness. In the work of Lundin (1953) the tissue N_2 elimination was determined from the accumulation of N_2 during O_2 breathing in a closed spirometer system. Because of technical difficulties involved in the collection and measurement of the small amount of N_2 given off from the tissues during O_2 breathing in a closed spirometer system, the importance of a further examination of this problem with another technique became evident. In the present study we have followed the elimination of N_2 during O_2 breathing by continuously measuring the changes in N_2 concentration of end-tidal air.

METHODS

The N_2 concentration in end-tidal air during O_2 breathing was measured with a Lundin–Åkesson nitrogen-meter. An improved model of the instrument was used which was some five times as sensitive as the previous version (Lundin & Åkesson, 1954). There was no loss

of stability and measurement of changes in N_2 percentages as low as 0·002% became possible. The experiments were carried out with the subject in a low-pressure chamber in order to achieve a further increase in the output of the instrument. With an unchanged ventilatory minute volume the relative percentage of N_2 in the expired gas is increased at low barometric pressure.

In preliminary experiments the pressure was lowered in two stages, with a period of 90 min at 380 mm Hg followed by 150 min at 190 mm Hg. In this way the N_2 sensitivity would be increased during the later period of slow N_2 elimination at 190 mm Hg, after the rapidly eliminated N_2 had been washed out at 380 mm Hg. Although five experiments on three subjects were carried out in this manner, calculation of the results proved difficult because of the necessity for correction for the slight N_2 contamination, 0·3%, of the O_2 inhaled, and its increased concentration because of the O_2 used by the body. However, it became evident that the output and stability of the N_2 meter was sufficient to permit the use of a barometric pressure of about 285 mm Hg (or a simulated altitude of 7500 m) which gave a relative increase in the concentration of expired N_2 of

$$\frac{\text{Barometric pressure}}{285}.$$

In order to achieve a further increase in the sensitivity of the N_2 meter the CO_2 and water vapour were absorbed from the expired gas before it reached the N_2 meter. In this way the following increase in sensitivity was obtained,

$$\frac{\text{Barometric pressure} - pCO_2 - pH_2O}{285 - pCO_2 - pH_2O};$$

so that a given number of N_2 molecules occupied about 3·5 times the volume at ground level. The ventilatory minute volume being the same, this therefore means a 3·5 times as big a N_2 percentage in the CO_2-, H_2O-free end-tidal air. With the more sensitive meter the total increase in sensitivity was about 17·5 times that previously available and it became possible to read changes in the analysed N_2 percentages with an accuracy of about $\pm$0·0006%. The altitude of 7500 m was chosen because there is very little likelihood of the occurrence of decompression sickness in a resting subject at that altitude.

Procedure

The subject reclined in a comfortable chair and breathed room air for a resting period of 20–30 min, during which the experimental arrangements were tested, the subject being connected to an ordinary air-craft pilot's respiratory demand valve through which he breathed pure O_2. The pressure within the chamber was then lowered with the subject breathing normally. No attempt was made to wash out the lung N_2 by over-ventilation. After 5–6 min the observer outside read the N_2 percentages in the CO_2-, H_2O-free end-tidal air, which was sampled with a modified Otis–Rahn pump (Rahn & Otis, 1947). With this system the air was taken through a container filled with granulated soda-lime to the pumping bladder. A needle valve was placed between the soda-lime container and the pump through which the air to be analysed was sucked to the N_2 meter. This needle valve could be closely controlled from outside the chamber with the help of a servo mechanism. When the chamber pressure had fallen to 285 mm the needle valve was adjusted until the pressure in the ionization chamber of the N_2 meter became 2 mm.

Before the sampled gas entered the ionization chamber it traversed a cooling drier consisting of a Dewar bottle filled with 96% ethanol and solid CO_2. When the gas passed through a glass tube immersed in this solution its H_2O pressure fell to practically zero.

The N_2 content of the end-tidal air was followed for 240 min. During the first 20 min a reading was taken every 60 sec, and after that time every fifth minute. In a few experiments, with subjects where the fall in nitrogen content of end-tidal air was very uniform,

readings were taken every 10 min after an initial period of 100 min. After 4 hr the experiment was terminated and the subject restored to normal barometric pressure.

In order to avoid leakage of N_2 into the breathing system the subject sat with head and shoulders in a tent of plastic film through which O_2 was streaming. The exhaled air, after passing through an ice-filled copper cooler, went out into the top of this tent. The demand valve and its connexion, which were inside the tent, were surrounded by a small polyethylene bag through which O_2 was streaming from a container outside the low-pressure chamber. After having passed through the bag the O_2 flowed out into the first-mentioned tent. In this way the head of the subject, after a few minutes, was surrounded by an almost pure O_2 atmosphere. This minimized the danger of leakage which, in our experience with the

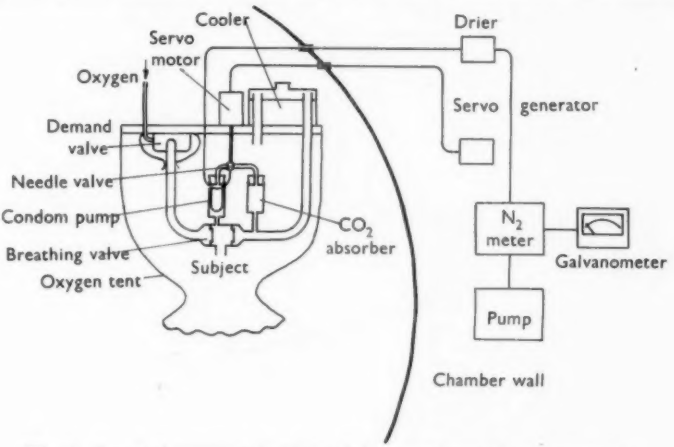

Fig. 1. Semi-schematical drawing of the experimental arrangements.

system, was most likely to occur in the demand valve system, or somewhere along the contact of the soft rubber face mask with the face of the subject. The softness and the seating properties of this mask were arranged to depend upon the gas pressure in a tube which formed its rim, and the subject could control this pressure by means of a rubber balloon. This pump bladder was connected to the mask through a tube which passed through an opening in the wooden disk which made up the roof of the tent. The subject was accustomed to the control of the rim pressure by several blank experiments in the low-pressure chamber. However, even with such precautions some leakage might occur, particularly after some hours, but this was detected on the recording meter and the subject advised to adjust the face mask. A small leakage did not disturb the shape of the elimination curve. After 1–2 min the deviation from the curve disappeared and representative readings could be obtained as before.

Figure 1 is a semi-schematic drawing of the experimental arrangements. The inspired O_2 was analysed for N_2 content with the help of the N_2 meter, and the readings obtained were corrected for the influence of increase in concentration at 7500 m brought about by the O_2 consumption. This correction was made in the following way:

$$N_2 \text{ meter readings} \times \frac{\text{Pressure at 7500 m}}{\text{Pressure at 7500 m} - 38/0{\cdot}8}, \qquad (1)$$

where 38 is the average end-tidal CO_2 pressure previously measured in our subjects, and 0·8 is the assumed R.Q. The value obtained was subtracted from the registered values without transformation into percentages since there was no reason for performing such a

transformation when the alveolar ventilation was not measured. The scale on the N_2 meter was linear in the range of measurements and the values obtained were thus proportional to the percentage values. The corrected values were plotted against time, with time in minutes as the linear abscissa and the N_2 meter readings as the logarithmic ordinate. If the ventilation for each subject was the same throughout an experiment, then this curve should give a picture of the N_2 elimination rate during O_2 breathing for each individual.

The curves obtained were treated in the same way as the N_2 elimination curves in previous work (Lundin, 1953). This means that the last part of the curve, representing the slowest eliminated N_2 fraction and forming a straight line, is projected to the left, and this straight line is used to separate out other N_2 fractions representing tissues with more rapid elimination rates.

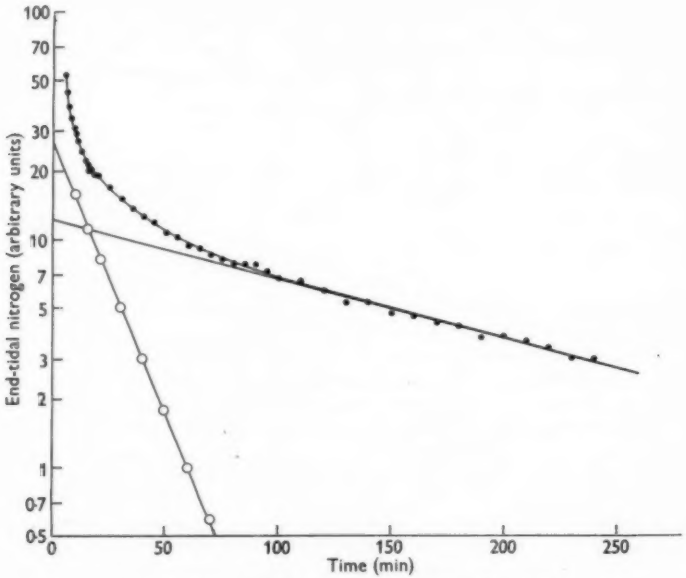

Fig. 2. Nitrogen elimination curve. Semi-log. scale.

The nitrogen value R_t at any point of the nitrogen elimination curve can be expressed:

$$R_t = R'_0 e^{-k_1 t} + R''_0 e^{-k_2 t}, \tag{2}$$

where R'_0 and R''_0 are the values for N_2 at time zero for the component curves, t is time in minutes, k_1, k_2 are time constants, or fraction of N_2 eliminated per minute. The relative amount of N_2 under each curve component is given by

$$N = \frac{R_0}{k}. \tag{3}$$

RESULTS

Thirty experiments were made on six subjects, the number of experiments on each subject varying between 2 and 11. The treatment of the semi-logarithmic curves described above permits the isolation of two com-

ponents. The rapid component reported in our earlier work (representing highly vascular tissues) could not be obtained because the N_2 elimination was not measured during the first 6–8 min. The average values for each subject are summarized in Table 1.

From the table it can be seen that the more rapid phase has mean k values between 0·046 and 0·061 and the slow phase k values between 0·0047 and 0·0079, indicating that the first phase represents tissues which eliminate between 4·6 and 6·1 % of their N_2 content per minute, and the

TABLE 1

Subject	Age	Weight (kg)	Height (cm)	No. of experiments	R_{0_M}	k_M	N_2 eliminated (arbit. units)	Desaturation half-time (min)
				Fraction *M*				
L.Å.	33	79	182	5	27·5	0·055	500	13·0
B.E.	28	59	165	5	28·0	0·061	460	12·9
G.L.	45	80	183	11	30·0	0·051	588	13·5
I.L.	16	60	163	4	11·7	0·050	234	13·5
S.A.	16	53	163	3	24·0	0·046	520	15·0
L.D.	22	60	176	2	25·0	0·051	490	13·5
				Fraction *F*		k_F		
L.Å.	33	79	182	5	12·1	0·0059	2050	120
B.E.	28	59	165	5	12·8	0·0048	2670	145
G.L.	45	80	183	11	13·5	0·0047	2870	150
I.L.	16	60	163	4	8·0	0·0051	1570	135
S.A.	16	53	163	3	7·1	0·0079	900	90
L.D.	22	60	176	2	7·6	0·0059	1290	120

Fraction *M*: the phase of more rapid elimination of N_2 during O_2 breathing, presumably from muscle. Fraction *F*: the phase of slowest elimination, presumably from fat. N_2 eliminated given by $\frac{R_{0_M}}{k_M}$ and $\frac{R_{0_F}}{k_F}$, respectively. k: N_2 elimination time constant of equation (2). R_0: nitrogen elimination rate at time zero of equation (2), in arbitrary units.

second phase tissues with a N_2 elimination rate of between 0·47 and 0·79 %/min. The elimination half-times vary between 12 and 15 min for the faster and between 90 and 150 min for the slower phase.

There is no relationship between age and elimination rate for the slow phase: but the subjects with the relatively largest N_2 volumes from this phase, namely G. L., B. E. and I. L., have the slowest elimination rates and the subjects with the smallest relative amount of N_2 the most rapid elimination rate. The rate of N_2 elimination from the 'muscle' phase is not obviously related to age. The youngest subject had a somewhat slower elimination rate than the other subjects but was the only one who occasionally slept during the experiment and it is probable that his metabolism was more basal than that of the others. Compared with our previous

results these experiments give the same values for the N_2 elimination rates from the two slowest components of the elimination curve. There is a 10:1 relationship between relative elimination rates for the two fractions labelled 'muscle' and 'fat' nitrogen.

DISCUSSION

Earlier unpublished investigations with the closed-circuit technique by W. M. Boothby & G. Lundin have shown that the elimination rate of N_2 during O_2 breathing is the same at ground level in Lund, and at a simulated height of 7500 m in a low-pressure chamber. These findings indicate that the cardiac output and vascularity of the body tissues remain unchanged. From earlier work (Lundin, 1953) we have seen that the ventilatory minute volume and O_2 consumption remained fairly constant during 4 hr of O_2 breathing. In the present series of open-circuit measurements the subject was quite as comfortable as before and therefore we have assumed that the ventilation and O_2 consumption have been constant during the whole experiment. The N_2 percentage in the end-tidal air should then reflect the amount of N_2 and the blood flow through the body tissues. In our earlier series of experiments (Lundin, 1953) the amount of N_2 from the two phases showed a good relationship to probable amount of muscles and fat of the subjects. In this series of experiments a direct calculation of probable amount of fat and muscles was not possible because the values were given in relative figures. However, an indirect calculation of fat and muscle of the subjects can be made if we assume that the two curves represent nearly all N_2 from the muscle and fat tissues. The area under each component of the curve should be proportional to the amount of N_2 given off.

From anatomical observations we know that about 50 % of the weight of the lean body mass (= total body minus fat) consists of muscle tissue (Vierordt, 1906). From the investigations of Campbell & Hill (1933), among others, we know that the solubility of N_2 per unit weight in fat tissue is about six times that in tissues such as muscle and blood, with high content of water and negligible fat. We can then construct the following equations for the determination of the absolute amount of fat and muscle tissue.

$$(W-y)\times 0{\cdot}50 = X. \tag{4}$$

Where W is total weight, X weight of muscles and y weight of fat, in kg, and $W-y$ thus equals the lean body mass. X is thus the muscle weight.

But

$$\frac{X}{y} = \frac{N_M}{\frac{1}{6}N_F}, \tag{5}$$

when N_M is the surface area of the curve representing the rapid 'muscle'

fraction of the N_2 elimination and N_F is the surface area of the 'fat' curve determined from equation (3).

The amounts of muscle M and fat tissue F in the subjects, calculated from the expression (4) and (5) are given in Table 2.

The values accord fairly well with what could be expected from the habitus of the subjects, and from other observations (Vierordt, 1906; Keys & Brozek, 1953). On subjects L. Å., B. E. and G. L. specific weight

TABLE 2

	Muscle (kg)*	Fat (kg)*
L.Å.	29·5	20·1
B.E.	20·0	19·2
G.L.	28·5	23·1
I.L.	19·3	21·5
S.A.	23·2	6·7
L.D.	24·6	10·8

* Weights are calculated from equations (4) and (5).

determinations were made by Dr W. von Döbeln by weighing the subject in air and in water. The weight of fat, F, in these subjects was calculated from the equation:

$$F = 4{\cdot}5 \times V - 4{\cdot}09\,W, \qquad (6)$$

where V is the volume of the subject corrected for the lung volume and W is the weight of the subject. The weight of fat was for L. Å. = 20·8, B. E. = 18·7 and G. L. = 22·8 kg. These values, together with the fact that the k values in this series of experiments are very much the same as in our former experiments, strongly indicate the reliability of the measurements and probability of the assumptions that the two components represent mainly nitrogen from muscle and fat tissue.

It has been shown by Jones (1951) and by Roughton (1952) among others that the circulation rate through the tissues is proportional to the measured N_2 elimination rate (expressed in terms of the factor k) and also proportional to the solubility of N_2 in tissue when compared with the solubility in blood. Thus from the present experiments the blood flow through muscle tissue should be between 46 and 61 ml./min/l. muscle tissue, and for fat between 24 and 40 ml./min/l. fat tissue.

Do these N_2 elimination values provide any information concerning the origin of the N_2 bubbles which have long been known to give rise to decompression sickness? A final answer to this question probably cannot be obtained from experiments of this nature; but the rapid elimination rate of N_2 from the muscles does not fit in with Jones's theory of the muscles as the place of formation of the N_2 bubbles. Also, the similarity in elimination rate of the muscle phase for younger and older subjects does not

agree with the observations that older subjects are more prone to develop 'bends' and other symptoms at altitude, and that a much longer period of pre-oxygenation is required in older subjects than in younger to avoid the bends.

One observation made in this series of experiments accords well with the assumption that fat tissue, or tissue with the same N_2 elimination rate as fat, is the place where, at least in resting subjects, the bubbles causing bends originate. Two preliminary experiments, performed in two stages, on subject G. L. (with 60 min at 5500 m) had to be stopped because of the symptoms of bends at a pressure of 190 mm. Subject G. L. (who has an experience of several hundred low-pressure chamber runs to different altitudes) is prone to develop bends at 30,000 ft. (9140 m) when the reduction of pressure is rapid. If it is assumed that the bubbles causing the bends are formed on the venous side of the circulation (Behnke, 1951), then the gas pressure in the venous blood at ground level is about 707 mm Hg (573 mm N_2, 47 mm CO_2, 47 mm H_2O and 40 mm O_2). At 30,000 ft. the barometric pressure is 226 mm, a 3·1:1 ratio between the gas pressure at ground level and at this altitude. In the present experiments, with the values obtained for N_2 elimination rate from the 'fat' of subject G. L. it is found that 60 min pre-oxygenation gives a 24 % decrease of N_2 content in the 'fat' tissue. The subject should then have a reduction of N_2 pressure in the venous blood from this tissue of $573 \times 0{\cdot}24 = 137$ mm. The pressure relationship between gases in the venous blood and barometric pressure 190 mm should then be

$$\frac{707-137}{190} \quad \text{or} \quad 3:1$$

which is very much the same as the relationship between ground level and 30,000 ft. with zero pre-oxygenation time. Bends would thus be expected. There is also an old observation that bends in resting subjects are rare at heights below 30,000 ft.

A prolongation of pre-oxygenation to 90 min would give a reduction of 'fat' N_2 of 34 %, or a gas tension in venous blood of

$$707-(573 \times 0{\cdot}34) = 512 \text{ mm},$$

a ratio to final pressure 190 mm, of 2·7:1. This would be equivalent to a rapid reduction of pressure up to 26,000 ft. (7925 m) without pre-oxygenation. No symptoms of decompression sickness were observed in any of the five experiments made under this schedule, nor in any of the other twenty-five experiments where this pressure ratio at the beginning of the stay at altitude was about 707:285 = 2·5.

From these theoretical considerations it seems reasonable to assume a close relationship in desaturation time between the tissues producing the

N_2 bubbles of bends and the fat tissue. The well known fact that fat subjects are more apt to develop decompression sickness strongly suggests that the bends tissue is fat or a tissue which changes its rate of elimination of N_2 in proportion to the amount of body fat.

SUMMARY

1. Nitrogen content of end-tidal air during oxygen breathing has been followed by means of a sensitive nitrogen meter.

2. The nitrogen desaturation rate of the body could be measured.

3. The desaturation curve could be separated into exponential fractions.

4. These fractions can be used to calculate the amount of fat and muscle tissue in the subjects.

5. The blood flow through these tissues can be calculated.

6. A relationship between nitrogen desaturation rate of fat tissue and decompression sickness seems to be probable.

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THE INFLUENCE OF HISTAMINE INHALATION ON THE PULMONARY DIFFUSING CAPACITY IN MAN

By A. BOUHUYS*, J. GEORG, R. JÖNSSON, G. LUNDIN and S.-E. LINDELL†

From the Medical Department B, Rigshospitalet, Copenhagen, Denmark, and the Institute of Physiology, University of Lund, Sweden

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Inhalation of a histamine aerosol in sufficient concentrations produces an increased degree of unequal distribution of inspired gas in the lungs. A relationship has been found between this change in the pulmonary ventilation and the relative amounts of histamine inhaled (Bouhuys, Jönsson, Lichtneckert, Lindell, Lundgren, Lundin & Ringquist, 1960). The results of this study also indicated that a variable amount of air might be trapped behind occluded bronchi in some experiments after histamine inhalation. Measurements with a body plethysmograph at the Pneumoconiosis Research Unit, Cardiff, showed an increase of the thoracic gas volume, measured at the end of a normal expiration, after histamine inhalation (A. Bouhuys, J. C. Gilson and M. McDermott, unpublished experiments), which also suggests the occurrence of air trapping. There is thus evidence to show that histamine inhalation may result in a decrease of the volume, and presumably of the surface area, of the gas-exchanging parts of the lungs. Recently, however, it has been reported (Lewis, Hayford-Welsing & McGinty, 1959) that no significant changes in the diffusing capacity of the lungs for carbon monoxide occur after induction of bronchoconstriction by histamine inhalation. This work was done both with a breath-holding CO method and with a rebreathing method. In the experiments to be described, the effect of histamine inhalation has been studied by a steady-state carbon monoxide method for determining the diffusing capacity of the lungs. The respiratory dead space has also been calculated from the figures obtained.

* Present address: Laboratory of Clinical Physiology, University Hospital, Leiden, The Netherlands.

† Present address: Laboratory of Clinical Physiology, Sahlgrenska Sjukhuset, Gothenburg, Sweden.

METHODS

Subjects. These were four male laboratory workers in good health. One of them (B) gave a history of bronchial asthma, but was symptom-free at the time of study. He was much more sensitive to inhaled histamine than the other subjects. The subjects were seated in a comfortable chair and inhaled the aerosol of a histamine solution (or, in the control experiments, of water) for 2 min. Immediately afterwards, they were connected to the bag containing the CO mixture, and a measurement of $D_{L_{CO}}$ was obtained.

Inhalation of histamine aerosol. The aerosol was delivered at a controlled constant flow rate by a nebulizer connected to a compressed-air cylinder. The duration of inhalation (2 min) and the flow rate of the aerosol were the same in all experiments. The concentrations of histamine in the nebulized fluid were chosen according to the subject's sensitivity, which was known from a previous study (Bouhuys *et al.* 1960). The aim was to give a dose of histamine sufficient to produce some effect on the bronchi but too low to produce marked discomfort.

Measurement of CO diffusing capacity ($D_{L_{CO}}$). The method described by Bates, Boucot & Dormer (1955) was used, with the following modifications. The minute volume of breathing was determined by connecting a wet gas meter to the box of a bag-in-box system, the CO mixture (0·14 % CO in air) being inspired from the bag. Expired gas was not collected during the experiment. For sampling of end-tidal expired gas, a relay system similar to that described by Bates (1958) was used. Breathing valves with minimal dead space were used. To minimize as much as possible the effects of back pressure of CO in pulmonary capillary blood, all subjects breathed pure oxygen for about 10 min in the interval, of at least 1 hr duration, between experiments of the same series.

Respiratory dead space. The personal dead space was calculated by means of Bohr's formula from the inspired, mixed expired, and end-tidal expired CO concentrations.

RESULTS

The minute volume of breathing did not change consistently after histamine inhalation (Table 1). With some exceptions, the CO uptake was lower, and the end-tidal CO partial pressure higher, after histamine inhalation. The diffusing capacity for CO was in all subjects lower after histamine than in the control experiments (Table 1).

In Table 2 these results are compared with the effect of inhaled histamine on the functional residual capacity (*FRC*) and the lung clearance index (*LCI*) of three subjects (N_2 wash-out method; Bouhuys *et al.* 1960). In these subjects the *FRC* decreased after histamine inhalation. The effect of histamine inhalation on the N_2 wash-out from the lungs was determined during the weeks preceding the present experiments. The last column of Table 2 gives figures for $D_{L_{CO}}$ which have been corrected to the average control value of the *FRC* in each subject, assuming proportionality between $D_{L_{CO}}$ and *FRC* (see Discussion). It has also been assumed that the histamine effect on the *FRC* was the same during the CO experiments as during the N_2 wash-out experiments. The decrease of $D_{L_{CO}}$ after histamine inhalation is much less evident in these corrected figures. There were no consistent changes in the volume of the personal dead space in any subject (Table 3).

TABLE 1. Effect of histamine inhalation on D_{LCO}

Subject	Time (min)	Histamine concentration (mg/ml.)	Minute vol. (l./min)	CO uptake (ml./min)	End-tidal CO pressure (mm Hg)	D_{LCO} (ml./mm/min)
B	0	0	7·54	5·81	0·360	16·1
	100	0	7·66	6·43	0·424	15·2
	195	0·05	5·42	4·44	0·480	9·3
B	0	0·05	7·00	5·67	0·422	13·4
	180	0	7·72	6·95	0·408	17·0
C	0	10	6·36	5·22	0·367	14·2
	80	0	5·58	5·19	0·297	17·5
	170	20	7·26	5·52	0·473	11·7
C	0	0	5·28	4·91	0·309	15·9
	60	20	6·28	4·52	0·471	9·6
G	0	0	7·92	6·81	0·289	23·6
	120	10	7·00	5·60	0·402	13·9
	210	0	6·96	6·54	0·332	19·7
G	0	20	6·70	5·83	0·408	14·3
	180	0	6·80	6·73	0·359	18·7
H	0	0	7·12	6·27	0·213	29·4
H	0	2·5	8·12	8·44	0·282	29·9
	100	5	8·72	8·02	0·374	21·4

Column 2: time from the beginning of control experiment. Column 3: histamine concentration in the nebulized fluid. Column 4: minute volume of breathing, corrected to body temperature, ambient pressure and saturated. Column 5: CO uptake corrected to 0° C, 760 mm Hg, dry. Column 6: partial pressure of CO in end-tidal expired air.

TABLE 2. Comparison between effect of histamine inhalation on gas distribution and on diffusing capacity

Subject	Histamine concentration (mg/ml.)	*FRC* (ml.)	*LCI*	D_{LCO} (ml./mm/min)	D_{LCO}, corrected (ml./mm/min)
B	0	1·860	10·2	16·1	16·1
	0·05	1·560	19·4	11·4	13·6
C	0	2·380	8·2	16·7	16·7
	10	2·210	14·6	14·2	15·3
	20	2·070	18·5	10·7	12·3
G	0	3·080	8·0	20·7	20·7
	20	1·970	12·8	14·3	22·4

Column 3: average values of the subject's *FRC* are given for the control experiments, and in others if more than one histamine inhalation had been done at the same dose level of histamine. Column 4: lung clearance index (index of nitrogen wash-out rate; see text). Column 6: values of D_{LCO} corrected for changes in *FRC*; see Discussion. Column 5: average values of D_L, from Table 1.

DISCUSSION

The diffusing capacity of the lungs (D_L) is a composite measurement, which reflects partly the size of the effective alveolar surface area (i.e. ventilated alveoli in contact with functioning capillaries), partly the properties of the 'pulmonary membrane' (i.e. the path from the alveolar surface to the intracorpuscular haemoglobin).

TABLE 3. Effect of histamine on respiratory dead space

Subject	Histamine concentration (mg/ml.)	% CO inspired	% CO alveolar	% CO expired	Tidal volume (ml.)	Dead space (ml.)	V_D/V_T
C	0	0·170	0·042	0·077	507	139	0·27
	0	0·171	0·044	0·084	406	128	0·31
	10	0·158	0·052	0·083	424	123	0·29
	20	0·166	0·067	0·099	331	106	0·32
	20	0·168	0·067	0·100	519	171	0·33
B	0	0·142	0·051	0·072	503	116	0·23
	0	0·161	0·060	0·082	589	130	0·22
	0	0·171	0·058	0·086	483	121	0·25
	0·05	0·169	0·068	0·094	361	94	0·26
	0·05	0·169	0·060	0·093	467	140	0·30
G	0	0·168	0·047	0·082	535	155	0·29
	0	0·151	0·041	0·072	566	158	0·28
	0	0·173	0·051	0·080	523	126	0·24
	10	0·159	0·057	0·084	500	130	0·26
	20	0·169	0·058	0·088	479	129	0·27
H	0	0·135	0·030	0·054	—	—	0·23
	2·5	0·159	0·040	0·062	677	122	0·18
	5	0·166	0·053	0·080	581	134	0·23

Columns 3–5: CO percentages corrected to 0°, 760 mm, dry. Columns 6 and 7: volumes corrected to body temperature, ambient pressure, saturated (*BTPS*). Column 8: dead-space:tidal-volume ratio.

The currently used methods of measuring D_L do not give identical results. The breath-holding method (Krogh, 1915; Forster, Fowler, Bates & van Lingen, 1954) tends to give higher values than the steady-state methods (Bates *et al.* 1955; Marks, Cugell, Cadigan & Gaensler, 1957). The breath-holding method is carried out with the lungs in an inspiratory position, whilst the steady-state method involves quiet breathing at the resting respiratory level. The difference in the lung volume during the experiment may at least partly explain the higher values found with breath-holding methods (Krogh, 1915; Marks *et al.* 1957; Marshall, 1958).

The decrease of steady-state $D_{L_{CO}}$ after histamine inhalation, found in the present study, may be explained by unequal distribution of inspired gas in the lungs, by a decrease of the *FRC*, or by changes in the pulmonary membrane which impede gas diffusion (e.g. interstitial oedema); or by a combination of some, or all, of these factors.

Changes in gas diffusion through the pulmonary membrane would be expected to affect D_L regardless of the method used for its measurement. As Lewis *et al.* (1959) found no change of breath-holding $D_{L_{CO}}$, this explanation of our findings seems unlikely.

The decrease of the *FRC* found in our subjects after histamine inhalation is very probably an expression of trapping of air behind occluded bronchi in some parts of the lungs. The discrepancy between the finding

of an unchanged breath-holding $D_{L_{CO}}$ (Lewis *et al.* 1959) and that of a decreased steady-state $D_{L_{CO}}$ (the present study) is most readily explained by the difference in the methods employed. In the inspiratory position (breath-holding method) the smaller bronchi are presumably opened, whereas air trapping may occur at the resting respiratory level (steady-state method). It may be assumed that Lewis *et al.* (1959) obtained degrees of bronchoconstriction which were at least as severe as the ones we induced. These authors reported a significant decrease of the vital capacity and of the forced-expiratory volume after inhalation, and one of us (A.B.) found that changes in these lung volumes are a less sensitive measure of the effect of inhaled histamine than the changes in *LCI* and *FRC* found with the N_2 wash-out method (unpublished experiments.)

To distinguish between effects of a decrease in *FRC* and of unequal distribution of inspired gas on D_L we tried to correct our D_L values for changes in *FRC* (Table 2). This correction was based on the assumption of proportionality between effective lung volume and diffusing capacity. The measurements of *FRC* and of D_L after histamine were not performed at the same time, and apart from this it is evident that such a correction has only value as a rough approximation. The corrected values of D_L only show a decrease after histamine doses which produced a marked degree of unequal distribution of inspired gas (subjects B and C; compare the values of the *LCI* in Table 2). Under these circumstances, the end-tidal CO concentration may no longer represent the mean alveolar CO concentration, which is likely to lead to an underestimation of D_L.

The personal dead space (Table 3) did not show significant changes after histamine inhalation. As the volume of the dead space is mainly influenced by that of the large airways this probably indicates that histamine acts mainly or only on the small bronchi. The same finding has been reported in cats by Folkow & Pappenheimer (1955).

SUMMARY

1. The diffusing capacity of the lungs ($D_{L_{CO}}$) was measured in four subjects with a steady-state CO method, before and after histamine inhalation.

2. $D_{L_{CO}}$ decreased after histamine inhalation in all subjects.

3. In some experiments this decrease was probably caused mainly by trapping of air in some parts of the lungs, in others an increased degree of unequal distribution of inspired gas in the lungs appeared to have an additional effect.

4. The respiratory dead space (Bohr's formula) did not change after histamine inhalation.

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THE EFFECT OF SEMISTARVATION ON ABSORPTION BY THE RAT SMALL INTESTINE *IN VITRO* AND *IN VIVO*

BY T. G. KERSHAW, K. D. NEAME* AND G. WISEMAN
From the Department of Physiology, University of Sheffield

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During a period of semistarvation there is a fall in the mass of body cells and a lowering of the basal metabolic rate (Wishart, 1934; Taylor & Keys, 1950; Grande, Anderson & Keys, 1958), the degree to which each change occurs depending upon the duration of the experiment and the severity of the dietary restriction. The loss of cellular mass and the fall in the metabolic rate depend upon the number of calories available, and provided that the caloric restriction is not too severe an equilibrium situation can be obtained. It was of interest therefore to investigate the absorptive ability of the small intestine during semistarvation, as it seemed a vulnerable organ in view of its great demands on the metabolic pool for the frequent replacement of its epithelial lining (Leblond & Stevens, 1948; McMinn, 1954).

We have found that after a period of semistarvation sufficient to cause a loss of about 20% of the initial body weight, there is an enhanced rate of disappearance of both glucose and L-histidine from the small intestine of the rat *in vivo*, and that *in vitro* the small intestine from semistarved animals can transport both these substances against a concentration gradient to a greater extent than can the small intestine of rats fed on an '*ad libitum*' diet. A preliminary report has been given by Neame & Wiseman (1959).

METHODS

Animals and diet. Male albino rats of an inbred strain were used, and were kept individually in separating cages with free access to water. The food used throughout was Diet 86, purchased from The North-Eastern Agricultural Cooperative Society, Ltd., Bannermill Place, Aberdeen, its composition being: soluble carbohydrate 53·4%; protein, 20·0%; fat, 3·8%; fibre, 3·3%; ash, 5·2%; moisture, 14·3%.

All animals were inspected every day and the dietary regimen was as follows:

Group *A* was fed *ad libitum*.
Group *B* was fed 5 g food per rat per day for 5 days.
Group *C* was fed 5 g food per rat per day for 9 days.
Group *D* was fed 5 g food per rat per day for 9 days, and then fed *ad libitum* for 24 hr.
Group *E* was fed 5 g food per rat per day for 9 days, and then fed *ad libitum* for 3 days.
Group *F* was fed 5 g food per rat per day for 9 days, and then fed *ad libitum* for 7 days.

* Present address: Department of Medicine, University of Otago, Dunedin, New Zealand.

In vitro experiments

Animals from all groups were used.

Preparation of sacs. The animal was killed by a blow on the head, the abdomen and thorax immediately opened, and the heart incised. The small intestine was then removed and everted, and sacs prepared as described by Wilson & Wiseman (1954). Six sacs (3–4 cm in length) were obtained from each small intestine, and their initial and final volumes were measured as described by Wiseman (1957). Occasionally the serosal volume of a sac decreased: such sacs were discarded.

Experimental procedure. The sac, filled with a known volume of amino-acid-glucose solution (0·5–1·0 ml.), was placed in a 150 ml. Erlenmeyer flask containing 20 ml. of the same solution as that used for filling the sac. The air in the flask was then replaced with a gas mixture of 5 % CO_2, 95 % O_2 and the flask tightly stoppered. The flask and its contents were kept at 37° C and continuously shaken for 1 hr by the use of a Warburg bath (80 oscillations/min, amplitude 5 cm). The sac was then removed from the flask, its surface drained, and its fluid contents recovered and weighed. Samples of initial and final serosal and mucosal fluids were analysed for amino-acid and glucose concentrations. A short length of thread ligature left at one end of the sac greatly facilitates the removal of the sac from the flask.

Concentration ratios. The concentration ratio referred to in the results is the ratio of the concentration of L-histidine or glucose in the serosal fluid (inside the sac of everted intestine) to the concentration in the mucosal fluid (outside the sac).

Amounts of glucose and L-histidine transported. These are expressed as the amounts transported into the serosal fluid per 100 mg dry weight of sac during the experimental period.

In vivo experiments

Animals from groups *A* (controls), *B* and *C* were used for the study of glucose absorption, and from groups *A* and *C* for L-histidine absorption. The animals were anaesthetized with pentobarbitone sodium B.P. 5 mg/100 g body wt., given intraperitoneally. As soon as anaesthesia had been induced the animal was placed on a warm operating table. The abdomen was opened by a mid-line incision and a transverse cut made in the lower duodenum and at the ileo-caecal junction, so that the whole of the small intestine could be washed out with bicarbonate saline solution (kept at 37° C). The duodenum was ligated about 1 cm distal to the transverse cut and the fluid contents of the intestine gently expressed. A loose ligature was placed around the lower ileum and a blunt needle, attached to a syringe containing glucose or L-histidine solution, was passed through the cut in the lower ileum and the ligature pulled tight over the needle shaft to prevent leakage of the injected 5 ml. test solution. As the needle was slowly withdrawn the ligature closed the intestine, which was then returned to the abdominal cavity. Absorption of the test solution was allowed to take place for a noted time, at the end of which the animal was killed by opening the chest and incising the heart. The small intestine was then removed, its surface carefully washed free from blood and its contents drained into a 50 ml. volumetric flask. The lumen was washed out with bicarbonate saline solution and the washings added to the 50 ml. flask, which was then filled up to the mark. The length of the small intestine was measured, care being taken to avoid stretching.

In some experiments the animals were killed as soon as the test solution had been injected into the intestine, and the latter washed out immediately.

Glucose and L-histidine solutions. D-glucose of Analar grade and L-histidine monohydrochloride of chemically pure grade were dissolved in bicarbonate saline solution (Krebs & Henseleit, 1932). In the *in vitro* experiments one solution was used containing 0·3 % glucose and 2 mM L-histidine. In the *in vivo* experiments either 0·4 % glucose was used or 1 mM

L-histidine with 0·3 % glucose. All solutions were gassed with 5 % CO_2 and 95 % O_2 before use.

Chemical estimations. Glucose was estimated by the colorimetric method of Nelson (1944), and L-histidine by the colorimetric method of Macpherson (1946).

RESULTS

In vitro experiments

Table 1 shows the effect of varying degrees of dietary restriction on the weights of the rats in the various groups. It should be noted that 5 g of food per rat per day represented about 20 % of that eaten per rat per day in the control group. The animals in groups *C–F* lost about 20 % of their starting weight during the period of dietary restriction, but remained otherwise apparently healthy. All the animals in groups *D*, *E* and *F* gained weight on being fed *ad libitum*, the animals in group *F* regaining their initial weight after 7 days.

TABLE 1. Effect of dietary regimen on body weight of rats used for *in vitro* experiments

		Nature of dietary regimen				
Group	No. of animals	No. of days restricted feeding (5 g/day)	No. of days subsequent feeding *ad lib.*	Wt. at start of dietary restriction (g)	Wt. at end of dietary restriction (g)	Wt. after subsequent feeding *ad lib.* (g)
A	6	(Controls. Fed *ad libitum* throughout: final weight 258 ± 16)				
B	6	5	None	269 ± 10	235 ± 8	—
C	6	9	None	285 ± 12	227 ± 8	—
D	6	9	1	277 ± 19	221 ± 17	248 ± 17
E	6	9	3	296 ± 26	217 ± 20	263 ± 23
F	6	9	7	285 ± 18	230 ± 14	295 ± 22

Values are means ± standard deviations.

Table 2 shows the effect of dietary restriction on the ability of the rat small intestine to absorb glucose and L-histidine against a concentration gradient. After 9 days of dietary restriction it was found that the small intestine was able to develop concentration gradients considerably greater than those developed by the control group, and that the quantities of glucose and L-histidine which could be so transported were increased. The small intestine of animals fed on a restricted diet for 5 days was able to transport amounts of glucose and L-histidine, and to develop concentration gradients, intermediate between those of the control group and those of animals which had been fed on a restricted diet for 9 days.

In order to investigate the rate of return of the intestine to its normal state, some animals were fed *ad libitum* for 1, 3 or 7 days after being on the restricted diet for 9 days. It will be seen from Table 2 that there was a

return towards normal in the intestine's ability to concentrate both L-histidine and glucose, even after as short a period as 24 hr on an unrestricted diet (group *D*). After 7 days on an unrestricted diet (group *F*) absorption of glucose and L-histidine by the intestine was virtually normal, while group *E* gave intermediate values. The sudden return to an unrestricted diet had no apparent ill effect on any rat.

The serosal volumes of the sacs from control animals increased by $30 \pm 4\%$, from animals on a restricted diet for 5 days by $45 \pm 5\%$, and from animals on a restricted diet for 9 days by $37 \pm 4\%$ (mean $\pm$ standard error of mean).

Table 3 shows the final concentration of L-histidine in the mucosal and serosal fluids from the sacs of everted small intestine. It will be seen that

TABLE 2. Effect of semistarvation on transport of glucose and L-histidine by sacs of everted small intestine *in vitro*. Initial L-histidine concentration in mucosal and serosal fluid was 2 mM; initial mucosal volume 20 ml.; initial serosal volume 0·5–1·0 ml.; length of sac 3–4 cm. Experimental period 1 hr. Temp. 37° C

Group	No. of sacs	Concentration ratios developed (serosal concn./mucosal concn.)		Amount transported/100 mg dry wt. of sac	
		Glucose	L-histidine	Glucose (mg)	L- histidine (μ mole)
A	35	$1{\cdot}44 \pm 0{\cdot}05$	$1{\cdot}81 \pm 0{\cdot}05$	$0{\cdot}80 \pm 0{\cdot}23$	$2{\cdot}81 \pm 0{\cdot}25$
B	34	$2{\cdot}38 \pm 0{\cdot}07$	$2{\cdot}74 \pm 0{\cdot}14$	$3{\cdot}78 \pm 0{\cdot}32$	$5{\cdot}31 \pm 0{\cdot}60$
C	32	$2{\cdot}63 \pm 0{\cdot}06$	$3{\cdot}17 \pm 0{\cdot}13$	$6{\cdot}59 \pm 0{\cdot}43$	$8{\cdot}30 \pm 0{\cdot}50$
D	33	$1{\cdot}99 \pm 0{\cdot}05$	$2{\cdot}74 \pm 0{\cdot}08$	$3{\cdot}94 \pm 0{\cdot}34$	$6{\cdot}80 \pm 0{\cdot}40$
E	35	$1{\cdot}94 \pm 0{\cdot}05$	$2{\cdot}18 \pm 0{\cdot}07$	$3{\cdot}15 \pm 0{\cdot}28$	$4{\cdot}49 \pm 0{\cdot}33$
F	36	$1{\cdot}52 \pm 0{\cdot}07$	$1{\cdot}77 \pm 0{\cdot}06$	$1{\cdot}24 \pm 0{\cdot}27$	$2{\cdot}59 \pm 0{\cdot}23$

Values are means $\pm$ standard error of means.

in all groups the mucosal fluid showed a fall and the serosal fluid a rise in the concentration of L-histidine. The serosal volume was small compared with the mucosal volume and this enabled a large increase in its L-histidine content to occur without causing more than a relatively small change in the L-histidine concentration of the mucosal fluid. Not all the L-histidine which disappeared from the mucosal fluid was transported to the serosal fluid. If we assume that this fraction was not metabolized, it is possible to calculate its average concentration on the basis of even distribution throughout the intestinal wall, although the actual concentration of L-histidine is presumably higher in the subepithelial space than in the subserosal space and the latter concentration must be higher than that in the serosal fluid. On the assumption that the concentration of L-histidine in the subserosal space is in near equilibrium with that in the serosal fluid, the estimates shown in Table 3 suggest that the L-histidine concentration in the subepithelial space of the sacs from animals on a restricted diet must be considerably higher than that in sacs from control animals.

TABLE 3. Effect of semistarvation on concentration of L-histidine in final mucosal and serosal fluids from sacs of everted small intestine, and estimated concentration in intestinal wall after 1 hr. Initial L-histidine concentration in mucosal and serosal fluid was 2 mM; initial mucosal volume 20 ml.; initial serosal volume 0·5–1·0 ml. Length of sac 3–4 cm. Temp. 37° C

Group	Final mucosal concn. (mM)	Estimated mean concn. in intestinal wall (mM)	Final serosal concn. (mM)
A	1·92 ± 0·02	3·8	3·40 ± 0·08
B	1·72 ± 0·02	9·7	4·66 ± 0·20
C	1·68 ± 0·02	10·5	5·32 ± 0·17
D	1·73 ± 0·02	8·3	4·70 ± 0·11
E	1·72 ± 0·02	9·7	3·72 ± 0·10
F	1·87 ± 0·02	4·0	3·26 ± 0·10

Values are means ± standard error of means.

In vivo experiments

Table 4 shows the effects of dietary restriction on the body weight and the length of the small intestine of the rats used for these experiments. Measurement of intestinal length presents a problem due to muscular contraction and the ease with which the intestine can be stretched. However, under standard conditions the results for the various groups showed relatively little scatter. It will be seen that the degree of loss of weight in these animals was comparable with that found for the animals used in the *in vitro* experiments. It can also be seen that restricting the diet of the

TABLE 4. Effect of dietary regimen on body weight and length of small intestine of rats used for *in vivo* experiments

Group	Test substance	No. of animals	Wt. at start of dietary restriction (g)	Wt. at end of dietary restriction (g)	Length of small intestine (cm)
A	Glucose	38 (Controls. Fed *ad lib.* throughout: final wt. 272 ± 4)			80 ± 5
B	Glucose	39	272 ± 3	229 ± 7	73 ± 5
C	Glucose	37	272 ± 4	209 ± 8	71 ± 4
A	Histidine	34 (Controls. Fed *ad lib.* throughout: final wt. 274 ± 3)			81 ± 6
C	Histidine	34	274 ± 3	207 ± 8	72 ± 6

Values are means ± standard deviations.

animals to 5 g of food per day for either 5 days or 9 days caused a significant decrease in the length of the small intestine. It may also be noted here that the intestine of animals on a decreased diet had a smaller diameter and was considerably thinner than that of animals fed *ad libitum*.

The effect of dietary restriction on glucose and L-histidine absorption is shown in Figs. 1 and 2 respectively. The amount of glucose absorbed was measured for experimental periods of up to 30 min. In the case of L-histidine it was decided to measure the amount absorbed at zero time, and after 10, 20 and 30 min, to enable a statistical evaluation to be made

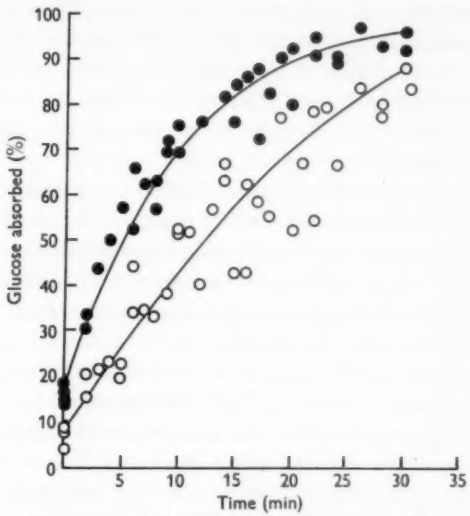

Fig. 1. Effect of dietary restriction on rate of absorption of glucose from whole of small intestine of rat *in vivo*. 5 ml. of 0·4 % glucose in bicarbonate saline solution introduced into each small intestine. ○, rats fed *ad. lib.*; ●, rats on restricted diet 9 days.

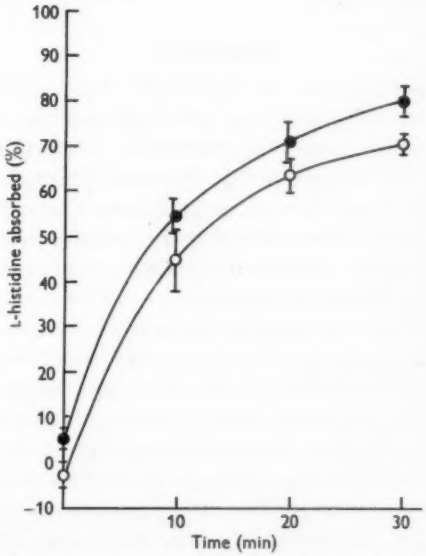

Fig. 2. Effect of dietary restriction on rate of absorption of L-histidine from whole of small intestine of rats *in vivo*. 5 ml. of 1 mM L-histidine with 0·3 % glucose in bicarbonate saline solution introduced into each small intestine. Ten animals in each group for estimation of absorption at zero time; eight animals in each group at 10, 20 and 30 min. Values shown are means and 95 % confidence intervals. Symbols as in Fig. 1.

of the results for each of these periods of absorption. The results plotted in Fig. 2 show means and 95% confidence intervals for each experimental period, i.e. it can be said with 95% certainty that the true mean lies within these limits. Testing the significance of the difference of the means, we found that at zero time $P < 0{\cdot}01$, at 10 min $P < 0{\cdot}02$, at 20 min $P < 0{\cdot}02$ and at 30 min $P < 0{\cdot}01$.

The semistarved animals absorbed better than control animals, the effect being more marked in the case of glucose. Animals on a restricted diet for 5 days absorbed glucose at about the same rate as those on a restricted diet for 9 days and have therefore been omitted from Fig. 1 for the sake of clarity. The amount of glucose absorbed by the semistarved animals after 10 min was about 60% more than that absorbed by the control animals, and after 20 min the corresponding value was about 27%. As almost all the glucose introduced into the intestinal lumen was absorbed in 30 min the difference between the two groups of animals became small at that time. For L-histidine the values at 10 and 20 min were 22 and 11% respectively. At zero time no L-histidine was absorbed by the control animals, whereas an immediate uptake of a small amount occurred in the restricted group. In the case of glucose, there was an immediate absorption of about 8% by the control animals and about 15% by the animals on a restricted diet.

DISCUSSION

Little critical work appears to have been done on the effect of semistarvation on intestinal absorption, although some incidental findings in rats deprived of food have been published. Thus Cori & Cori (1927), Horne, McDougal & Magee (1933), Marrazzi (1940), and Magee (1945) suggest that fasting causes a decrease in glucose absorption in rats *in vivo*, although Heller (1954) found that a 96 hr fast did not do so. However, these workers used very concentrated solutions of glucose and techniques which differed considerably from ours.

In the present paper the results of the *in vivo* experiments show that when 0·4% glucose solution is introduced into the lumen of the whole of the small intestine of semistarved rats, there is a disappearance of glucose at a rate greater than that found with rats fed *ad libitum*. Such an increase in the rate of disappearance of glucose could be due to an increased rate of utilization, increased storage by the intestinal wall, or increased absorption and transfer to the blood. The rate of glucose disappearance from the lumen in the *in vivo* experiments does not necessarily reflect the ability of the intestine to transfer glucose against a concentration gradient, but the latter can be easily measured by the use of sacs of everted small intestine *in vitro*. The results of such experiments show that the small intestine of

the semistarved animal can absorb glucose against its concentration gradient better than normal small intestine. An enhancement of absorption was also obtained when solutions of L-histidine were used, and although the increase in its rate of disappearance *in vivo* is less than that found for glucose the results will be seen to be statistically highly significant. It is interesting to note that the rate of disappearance of both glucose and L-histidine is enhanced *in vivo* in semistarved rats, despite the occurrence of a reduction in the length of the small intestine. From the *in vitro* experiments it is clear that the enhancement of transport of glucose and L-histidine is not due to a decrease in the ability of the intestine to absorb water. As was noted in the Results, the concentration of L-histidine in the subepithelial space must have been greater than that in the subserosal space of the sacs of everted small intestine. The rate of diffusion of L-histidine into the serosal fluid in such sacs will depend therefore on its subepithelial concentration and also on the barrier to diffusion presented by the intestinal wall. *In vitro*, therefore, the thinner intestinal wall found in the semistarved animals could conceivably exaggerate the difference between the absorptive powers of semistarved and control animals. In the case of absorption *in vivo*, however, the very close relationship between the villous epithelium and subepithelial capillaries is unaltered by dietary restriction and the enhanced rate of absorption of glucose and L-histidine in the semistarved animals *in vivo* cannot be exaggerated by a thinner intestinal wall. A more rapid rate of diffusion from the subepithelial space into the capillaries would, however, be brought about by the higher concentrations of glucose and L-histidine which are produced in this space in the animals on a restricted diet.

A sudden return to an unrestricted diet caused no ill effect after 9 days on a severely reduced diet. This is in agreement with the observation of Sun (1927) who found no ill effects on refeeding mice after starvation. These results are in contrast to the view that feeding *ad libitum* after a period of severe semistarvation may be fatal. Hehir (1922) has stated that in severe starvation there is a point of no return, despite provision of a diet adequate in calories. However, the results of the numerous investigations in Europe at the end of the 1939–45 war (Leyton, 1946; Mollison, 1946; Murray, 1947) would suggest that his view is incorrect in the absence of avitaminosis. The findings of Leyton (1946) are of interest in this respect as they show that in the absence of disease semistarved prisoners in German camps (who had lost at least 20% of their original weight) were able to eat immediately and without any ill effects an unrestricted diet provided that it had an adequate vitamin content.

SUMMARY

1. The effect of varying degrees of dietary restriction on the absorption of glucose and L-histidine by the small intestine of the rat has been studied *in vitro* and *in vivo*.

2. It was found that during a period of semistarvation some compensatory mechanism is brought into play which increases the ability of the small intestine to absorb glucose and L-histidine against a concentration gradient. The increase in active absorption disappeared after a few days on an '*ad libitum*' diet.

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